Cytotoxic Activity of Sphingosine-1-Phosphate against Human Triple-negative/ Basal-like Breast Cancer

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ABSTRACT

Breast cancer is one of the most common malignancy diagnosed in women and is the primary cause of cancer-related deaths in women worldwide. It is a heterogeneous group of diseases that have a different response, prognosis, and clinical outcomes. Estrogen, progesterone and HER2 negative breast cancer, known as triple negative breast cancer (TNBC), does not respond to hormonal therapy. Basal-like breast cancer (BLBC) has shorter overall survival rate among other subtypes. Tumors sharing both TNBC and BLBC are considered less responsive to currently available treatment. Chemoresistance to treatment has been a challenge in cancer biology and force investigation toward developing new targeted therapies, which selectively target specific subtypes. Sphingolipid metabolites have an important physiological role in determining cell fate. Sphingolipid metabolites, ceramide, sphingosine, and sphingosine-1-phosphate (S1P), are implicated in cancer. S1P exerts its functions via extracellular and intracellular targets. S1P synthesized inside the cell is exported outside and binds to G-protein coupled receptors, the sphingosine-1-phosphate receptors 1-5 (S1PR1-5). Although the intracellular function is not well defined, its suggested intracellular S1P promotes cell apoptosis. The S1P pathway has received great attention recently due its function in cell survival and death. This effect was reported to be concentration dependent.

In this research, I focused on S1P effect on nine TNBC/BLBC cell lines. I examined the *in-vitro* effects of S1P on apoptosis, proliferation, and cytotoxicity in triple negative/ basal-like breast cancer cell lines. Moreover, I studied the co-administration of S1P with currently used chemotherapeutic agents in these cell lines. Data show that S1P can selectively induce cell death in TNBC/BLBC cell lines at a specific concentration. In this research, I found that the

mechanism of cell death following treatment with different S1P concentrations was mainly due to apoptosis. Results show that S1P leads to cell shrinkage, rounding and detachment in the nine TNBC/BLBC cell lines. S1P combination with doxorubicin and docetaxel at different concentrations shows no beneficial effect of the combination compared to the chemotherapeuitc agent alone. In some cell lines, the combination showed a protective effect.

Further studies are required to determine the mechanism by which S1P induces cell apoptosis, inhibits cell growth, and demonstrates lack of responsiveness in combination studies.

ACKNOWLEDGEMENTS

I would like to acknowledge the support of my supervisor Jian Yang and advisory committee Jane Alcorn, Franco Vizeacoumar and Ed Krol.

DEDICATION

This thesis is dedicated to all my friends and family.

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LIST OF ABBREVIATIONS

ABCA1	ATP-binding cassette sub-family A
ABCC1	Multidrug resistance associated protein 1
ABCC7	Cystic fibrosis transmembrane conductance regulator; also known as CFTR
ATCC	American Type Culture Collection
BAD	Bcl-2-associated death promoter
BLBC	Basal-Like Breast Cancer
bFGF	Basic Fibroblastic Growth Factor
BRCA1	Breast cancer type 1 gene
BRCA2	Breast cancer type 2 gene
Ca2+	Calcium ion
CDK	Cyclin dependent kinase
CK	Cytokeratin
CNS	Central Nervous System
CO2	Carbone dioxide
DOC	Docetaxel
DOX	Doxorubicin
DNA	Deoxyribonucleic acid
EDG	Endothelial differentiation gene
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
eNOS	Endothelial nitric-oxide synthase
ER	Estrogen Receptors
ERK	Extracellular signal-regulated kinase
Fas	Tumour necrosis factor super-family 6 or apoptosis antigen 1
FITC	Fluorescein isothiocyanate
gm	Grams
HER2/neu	Human epidermal growth factor receptor 2
hr	Hours

ICH	Immunohistochemistry
IMP3	Small nucleolar ribonucleoprotein protein
JNK	Stress activated protein kinase
Ki67	Antigen KI-67
L	Liter
LDH	Lactate dehydrogenase
MAPK	Mitogen-activated protein kinase
µg	Microgram
µL	Microliter
µM	Micromolar
mg	Milligram
mL	Milliliter
mM	Millimolar
MMP	metalloprotease
mTOR	Mammalian target of Rapamycin
nM	Nonomolar
PBS	Phosphate Buffered Saline
pCR	Complete Pathological Response
PDGF	Platelet-derived growth factor
P-gp	P-glycoprotein transporter
PI3K/AKT	Phosphatidylinositol-3-kinase/ Serine-threonine protein kinase
PKC	Protein kinase C
PR	Progesterone receptor
PARP	Poly (ADP-ribose) polymerase
RNA	Ribonucleic acid
S1P	Sphingosine-1-phosphate
S1PRs	Sphingosine-1-phosphate receptors
SD	Standard deviation
siRNA	Small interference RNA
SphK	Sphingosine kinase
SM	Sphingomyelin

SMase	Sphingomyelin synthase
SPL	Sphingosine-1-phosphate lyase
TNF-α	Tumour necrosis factor alpha
TN	Triple Negative
TNBC	Triple Negative Breast Cancer
TNM	TNM Classification of Malignant Tumours
TP53	Tumour protein 53
VEGF	Vascular endothelial growth factor

1. LITERATURE REVIEW

1.1 BREAST CANCER

1.1.1 CLASSIFICATION

Breast cancer is the most common malignancy in women and the second most common cancer of all cancer incidence worldwide[1]. The term breast cancer includes a wide variety of tumors that affect the tissue of the breast. The molecular profiling of breast cancer reveals the heterogeneity of the disease[2]. Different subtypes identified by molecular profiling makes it more challenging to diagnose and treat these tumors. These molecular differences between the subtypes are reflected in clinical outcomes and responses to therapy[3].

The etiology of breast cancer is unknown. However, hereditary factors are considered the major determinant of individual risk[4]. Mutations in cancer susceptibility genes such as *BRCA1* and *BRCA2* can be used in the assessment of individuals at risk of developing breast cancer. Mutations in one or both genes increase the likelihood of developing breast cancer[5]. A defect in DNA repair or alteration in growth suppressor may also increase the risk of developing breast cancer[3]. The disease starts as a single cell transformation and the progression of a tumor depends on accumulation of these changes[3].

The breast consist of lobules, ducts, and adipose tissue[6]. The most common type of breast cancer is the ductal carcinoma, which begins in the cells of the ducts[7 8]. Breast cancer can also start in the lobules and other components of breast tissue. *In-situ* and invasive terms are used to describe how far the disease has spread. *In-situ* breast cancer is localized within the same tissue whereas invasive breast cancer implies that tumor has metastasized to surrounding tissue[9].

Breast tumors are classified using different systems. Each classification serves different purposes. Histopathology grading depends on the histological appearance of the tumor[10]. A grade of tumor compares the appearance of the breast cancer cells to the appearance of

normal breast tissue[10]. Low grade is well differentiated and high grade is poorly differentiated. Staging of malignant tumors (TNM) uses three factors to describe the prognosis of the tumor: the size of the tumor (T), lymph node involved (N), and metastasis (M)[11].

Receptor status is another classification of breast cancer that utilizes immunohistochemistry (IHC) assay to determine the presence or absence of receptors[12]. The three receptors assessed in breast cancer are estrogen receptor (ER), progesterone receptor (PR), and HER2/neu[13]. Molecular classification of breast tumors is based on comparing gene expression between normal and cancer cells and clusters breast carcinomas based on the gene expression[14].

1.1.2 BREAST CANCER MOLECULAR SUBTYPES

The current molecular classification of human breast carcinoma utilizes different markers to assess tumors and can be used as a diagnostic tool to determine the progression of diseases. Molecular classification leads to further understanding of breast cancer progression. The introduction of microarray technology divided breast cancer to different subtypes associated with different clinical outcome[15]. It has provided a broader definition of the disease as a group of malignancies that share the same origin but differ in their biology. DNA microarray clusters breast carcinomas based on the gene expression profiles shifting the treatment to more specific approach guided by certain gene expression patterns[16].

Gene expression profiling of few key proteins including estrogen receptors, (ER), progesterone receptor (PR), and human epidermal growth factor 2 (HER2) has led to the classification of breast cancer into five major molecule subtypes:

a) Luminal A, b) luminal B, c) human epidermal growth factor receptor 2 (HER), d) normal-like, and e) Triple negative/basal-like type[13]. Such a classification has helped in predicting the treatment and the prognosis of each type[17].

Luminal A subtype is estrogen receptor (ER) positive progesterone receptor (PR) positive, HER negative, and low expression of proliferation genes such as Ki67[18]. This subtype is the most common, comprises about 50% of all cases, and has the best prognosis[19]. Luminal B accounts for about 20% of all breast cancer cases and is considered to be more aggressive than luminal A[5]. Luminal B subtype is ER positive and PR positive and HER2 positive or HER2 negative with high expression of Ki67[19]. Luminal A and B subtypes have gene expression patterns similar to normal cells that line the breast ducts[20-22]. In general, luminal subtypes are known to have a better prognosis, high survival rate and low recurrence rate compared to other subtypes[19]. Hormonal therapy and targeted therapy have replaced chemotherapy for subclasses that overexpress the corresponding receptors. Treatment options of luminal subtypes is based on hormone receptor (HR) targeting such as Tamoxifene[23].

HER2 subtype is known for overexpression of human epidermal growth factor receptor 2 (HER2) genes[24]. This subtype of breast cancer comprises approximately 15% of all breast cancer cases[19]. HER2 subtype has a poor prognosis, and high recurrence rate. This subtype can be treated with targeted therapies such as trastuzumab (Herceptin)[24].

Normal-like breast tumors account for about 5–10% of all breast carcinomas[19]. These tumors are poorly characterized and have a gene expression characteristic of adipose tissue and basal cells[18]. Some studies refer to this subtype as an artifact due to the presence of some normal cells in the sample[25]. This subtype was reported to be triple negative (TN) but not basal-like, since it is EGFR and CK5 negative[19].

Triple-negative/basal-like breast cancer (TNBC) defined as a group of different breast tumors that share the same features but are yet still poorly characterized[2]. Triple-negative breast cancer (ER-, PR-, and HER2-) has poor prognosis[26]. Triple-negative and basal-like subtypes are often used synonymously, and the term basal-like and triple-negative terms are used interchangeably in the literature. However, not all triple-negative tumors are basal-like, and not all basal-like tumors are triple-negative[21]. In fact, triple-negative is considered as a subtype of basal-like cluster[27]. The unclear definition may be attributed to the origin of these tumors since they arise from the basal layer of breast duct[28]. Triple-negative and basal-like breast cancers show aggressive clinical behavior, poor clinical outcome, and high recurrence rates among all subtypes[29].

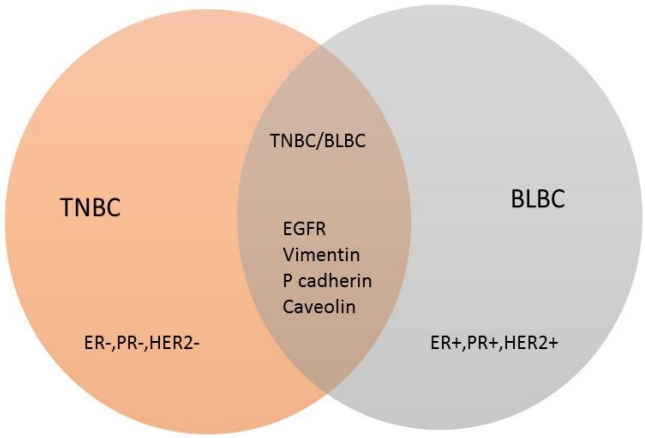

Figure 1: Overlap between TNBC and BLBC subtypes. Abbreviations: BLBC, basal-like breast cancer; ER, estrogen receptor; PgR, progesterone receptor; TNBC, triple-negative breast cancer

1.1.3 TRIPLE NEGATIVE BREAST CANCER

Triple negative breast cancers (TNBCs) are a group of breast cancers characterized by the absence of estrogen receptors (ER), progesterone receptors (PR) and HER[29]. Approximately 20% of breast cancer cases are triple negative[30,31]. TNBC is more common among young and African-American women[32].

Immunohistochemistry (IHC) methods are used for the assessment of this type of breast cancer. While there is an international agreement about the term triple negative the cutoff level is not yet standardized[15]. The American Society of Clinical Oncology guidelines for IHC testing requires that less than 1% of tumor cells with ER and PR negative to staining for the assessment of triple negative[12]. In this respect, a more standardized cutoff is required to improve diagnosis. Lacking (ER/PR/HER2) receptors make this subtype unresponsive to hormonal and antibody therapies. In clinical practice, TNBC is referred as basal tumors provided that TNBC do not express ER, PR, and HER2 proteins detected by IHC technique and hence therapeutic choices are based on IHC. However, the increased recurrence and a poorer prognosis provide supporting evidence of the diversity of TNBC[26,33].

1.1.4 BASAL-LIKE BREAST CANCER

Unlike TNBC, there is no international definition for Basal-Like Breast Cancer (BLBC)[34]. Human breast is composed of two cell layers, luminal cell and basal cell/myoepithelial[35]. Basal cells and luminal cells have different expression patterns of cytokeratins[36]. Myoepithelial cells express basal cytokeratins 5, 14, and 17, and myoepithelial associated proteins smooth muscle actin, S-100, and CD107. On the other hand, luminal cells express luminal cytokeratins (CKs) 7/8, 18 and 19[37]. BLBCs represent approximately 20% of

all breast cancer cases[2]. In addition to their expression of high molecular weight cytokeratins, they have high expression levels of P-cadherin, caveolin 1 and 2, nestin, CD44, and EGFR[38].

BLBC are mainly ductal carcinomas showing high mitotic index and of high grade[38]. Tumors of this type show a metastatic pattern different than luminal subtypes with predominance for lung and brain[3]. Around 80% of BLBCs lack the expression of the key receptors in breast cancer namely ER, PR, and HER2 receptors[19]. Therefore, a large overlap exists between TNBC and BLBC (Figure1). Despite the discordance between the two subtypes, the TNBC and BLBC terms are being used interchangeably in clinical settings.

Several studies attempted to establish a universal definition for the BLBCs. Nielsen *et al.* [38] proposed five markers to identify basal-like tumors. These markers, ER, PR, HER2, EGFR, and cytokratin5/6, have a high specificity and sensitivity to classify this subtype[36 38]. A worse prognosis is a common feature of BLBC subtype, and they have the same relapse rate as TNBC[26]. Identifying new therapeutic targets for TNBC/BLBC is critical because, despite their high response to chemotherapy, the prognosis is still poor.

1.1.4.1 EPIDEMIOLOGY

Epidemiologically, TNBCs and BLBCs are most common among younger patient, less than 50 years old and most prevalent in women of black decent[39]. Recurrence risk peaks between 1–3 years, which decrease after 5 years. Survival rates decline in both subtypes after 3-5 years of diagnosis. BLBCs are associated with poor relapse-free and overall survival. These tumors have large size at the time of diagnosis and are more common in younger patients[34].

1.1.4.2 MOLECULAR FEATURES OF TNBC/BLBC

TNBC and BLBC are not single diseases. Failure of the histopathological classification to define the subclasses of both types leads to the use of gene expression profiling to define these classes. While TNBC are negative for HER2, not all BLBCs are HER2 negative.

The basal subtypes of breast cancers are characterized by high expression of basal cytokeratin and genetic markers of basal epithelium[40]. Gene expression profiling of 172 TNBC tumors revealed that 71% were basal-like breast cancer, and 29% were non-basal[41]. Approximately 15%–45% of TNBCs have been shown to express ER and 14% of BLBCs to express HER2[39]. Approximately 90% of BLBC are triple-negative. TNBC immunohistochemical profile is characterized by expression of several markers including CK5, CK6, CK14, CK8/CK18, EGFR1, c-kit, and insulin-like growth factor receptor (IGFR)[42,43]. TNBCs have higher expression levels of CK5/6 and EGFR than luminal subtypes[26]. Poor prognosis is correlated with cytokeratin 17 and/or cytokeratin 5/6 expressing tumors. Basal cytokeratin expression is used as markers for basal tumors with 5/6 cytokeratin being considered as the most useful[44]. Insulin-like growth factor-II mRNA-binding protein 3 (IMP3), which is important in RNA trafficking and stabilization, promotes tumor cell growth. IMP3 is expressed during embryogenesis, and few studies reported expression in malignant tumors[45] but not in normal tissues. IMP3 expression in cancer makes it a strong candidate for consideration as a diagnostic marker[45]. The tumor suppressor genes, BRCAs, are involved in DNA double strand break repair[46]. There is strong evidence that *BRCA1* pathway is involved in the development of a basal-like, triple-negative subtype[42]. *BRCA1* mutant and TNBC share common clinical, pathologic, and molecular features. *BRCA1* tumors are of high-grade, high occurrence in younger women, and poor prognosis, which led to speculation that *BRCA1* is in triple-negative tumours[47]. The majority of *BRCA1*

breast tumors show expression of basal cytokeratins and EGFR. *BRCA1* mRNA expression levels were found to be diminished in TNBC[48]. The vast majority of BLBCs and TNBCs have P53 mutations[49]. These mutations could be an explanation for poor prognosis for both subtypes.

BRCA1 mutations are associated with high-risk breast cancer[46]. Defective *BRCA1*, which is critical for the repair of double stranded DNA, leads to accumulation of mutations in DNA repairing enzymes and enhanced tumor growth. *BRCA1* or *BRCA2* deficient tumors are sensitive to Poly-ADP ribosepolymerase-1 (PARP)[50]. PARP is a single strand break repair enzyme that when inhibited by (PARP-1) inhibitors leads to cell death due to the accumulation of these double strand DNA breaks.

1.1.4.3 CLINICAL FEATURES OF TNBC/BLBC

Aggressive clinical features of TNBC/BLBCs have been reported by several studies[37]. Poor clinical outcome and reduced disease-free survival compared to other types of breast cancer. A study carried by Dent *et al*. reported that the median time to death for patients with TNBC was 4.2 years[26]. The overall survival is shorter than for other breast cancers, and most deaths occur within the first five years after diagnosis[26 38]. Distant recurrence was reported to be higher than other breast cancer groups with local recurrence shorter in patients with TNBC than other breast cancer subtypes. Visceral, bone, and brain metastases have been reported to be higher in TNBC than other types of breast cancer[28]. All these results confirmed that TNBC has a relatively poor prognosis and less response to treatment.

1.1.4.4 TREATMENT OF TNBC

Despite the initial susceptibility of TNBCs to chemotherapy, their overall survival rates are low[51]. Compared to receptor positive subtypes, the risk of relapse is still high reaching peak after three years[52]. No specific treatments are available for TNBC with chemotherapy as the only option available for TNBC patients. However, efforts to identify targetable markers in TNBC are promising.

The effectiveness of chemotherapy in TNBC has been investigated by several studies[53-55]. Neoadjuvant chemotherapy response of TNBC was reported to be better than other breast cancer subtypes[30]. However, the prognosis is still poor. Taxanes, anthracycline and platinum-containing agents, such as cisplatin, are most frequently used in TNBC. However, recurrence rates are still high[48]. Taxanes containing an adjuvant or neoadjuvant regimens were found to be effective in early-stage disease[56]. Taxanes increased the diseases free survival by 87% as compared with cyclophosphamide among TNBC patients[56]**.** In general, there is no preferred single or combination chemotherapy specifically recommended for TNBC[57].

TNBC has been reported to have higher pathologic complete response (pCR) rates compared to HR positive breast cancer when treated with neoadjuvant chemotherapy receiving anthracycline or anthracycline and taxanes[58]. The response of TNBC to neoadjuvant therapy was significantly higher than other types of cancer. However, the overall 3-year progression free survival was reported to be less than other types demonstrating the poor prognosis in TNBC[54]. Neoadjuvant therapy with the platinum-based drug in TNBC patients with BRCA1 mutations showed that 90% of cases had complete pathological responses[44]. Studies with other cytotoxic drugs have been also conducted. Neoadjuvant cyclophosphamide showed higher pCR rate in TNBC patients as compared to luminal

subtypes[55]. Neoadjuvant ixabepilone (microtubules stabilizer) trials demonstrated a higher response to HR-negative than HR-positive tumors[59]. This study showed greater sensitivity for TNBC to ixabepilone than ER+ tumors.

Platinum-based therapies were reported to be effective in tumors with *BRCA1* mutations[60]. Accordingly, TNBC has demonstrated high pCR compared with non-TNBC patients[61]. However, platinum-based therapies showed no efficacy in BLBC tumors[62]. PARP is single-strand DNA break (SSB) repairing enzyme by base excision repair. Clinical studies using PARP inhibitors in *BRCA*-mutant patients are still ongoing. The combination of PARP inhibitor iniparib with carboplatin resulted in increased patient survival by five months[60]. Olaparib, a potent oral PARP inhibitor, was tested against TNBC tumors. Olaparib showed a very high response rate in TNBC tumors[63].

Cetuximab, an epidermal growth factor receptor (EGFR) inhibitor has been assessed in the TNBC[54]. Clinical trials on inhibition of angiogenesis in TNBC by monoclonal antibodies against the vascular endothelial growth factor (VEGF) receptor or VEGF receptor inhibitors are currently underway[64].Despite the result mentioned above, TNBCs demonstrate only modest response to treatment and remains a challenging disease to treat.

1.1.4.5 CHEMORESISTANCE IN TNBC

Chemoresistance is the major problem facing cancer treatment, which accounts approximately for 90% of treatment failure in metastatic cancer[65]. Tumors not only become resistant to the used drug but also may develop cross-resistance to other chemotherapies. As mentioned above, TNBCs lack the expression of ER, PR, and HER2 receptors that make them unresponsive to hormonal or anti-HER2 agents. Understanding the diversity of TNBC

/BLBC and their molecular characteristics will help in bypassing the chemoresistance developed by these tumors.

Previous studies have indicated several targets for drug resistances in TNBC[51 56]. ATP-binding cassette transporters (ABC transporters) are among most thoroughly investigated targets in TNBC chemoresistance. Transporters affect the ability of chemotherapeutic drugs to reach its proposed target. P-glycoprotein (P-gp), multidrug resistance-associated protein (MRP), and breast cancer resistance protein (BCRP) are the most common transporters involved in drug resistance in TNBC[66]. Decreased intracellular doxorubicin accumulation in TNBC was attributed to overexpression of BCRP[66]. P-gp was observed to efflux a wide range of chemotherapeutic agents[67].

Another mechanism of resistance to chemotherapy is the overexpression of β-tubulin III subunit which was found to be correlated to paclitaxel resistance[68]. β-Tubulin III together with α-tubulin is responsible for the formation of microtubules which is the target for paclitaxel related cell death[69]. Overexpression of β-tubulin III in TNBC[51] explains why resistance to paclitaxel exists in some TNBC tumors. Mutations in DNA repairing enzymes such as topoisomerase II was correlated with chemoresistance to anthracyclines[51]. Alteration in apoptosis regulating genes such as p53 and caspase-3 were correlated with chemoresistance of tumors to tubulin inhibitors [70 71].

Given these data, we can affirm that TNBC/BLBC subtypes do not all respond in the same magnitude to chemotherapy treatment. The response depends largely on gene expression of the targets mentioned above.

1.2 SPHINGOLIPIDS

1.2.1 INTRODUCTION

Sphingolipids were first identified in the 1870s in brain extracts and were named after the Greek mythological creature, the Sphinx, because of their enigmatic nature[72]. Sphingolipids constitute a class of lipids defined by their eighteen carbon amino-alcohol backbones that are synthesized in the endoplasmic reticulum[73]. In addition to their supportive role in the cell membrane in eukaryotic cells, sphingolipids play a pivotal role as signaling molecules. Sphingolipid metabolites, ceramide, sphingosine, and shingosine-1-phosphate (S1P), are the major signaling sphingolipids[74 75] and have been identified as a determinant of cell fate, which regulate cell proliferation, survival, and apoptosis[76].

The potential roles of sphingolipid derivatives have been described in different systems such as cardiovascular, immune, and nervous systems[77]. However, alteration in their levels were implicated in many pathophysiological disorders such as cardiovascular diseases, diabetes, inflammatory, and infectious diseases[78].

Sphingolipids' role in cancer has received significant attention recently. The growing evidence of sphingolipid pathway involvement in carcinogenesis has been reported by several studies[79-82]. The role of sphingolipid signaling in breast cancer subtypes was reported by expression levels of certain enzymes involved in the metabolic pathway of sphingolipids. These results reflect the impact on prognosis and resistance to chemotherapy. Sphingosine -1-Phosphate is the end metabolite of interest that draws researcher's attention as a signaling molecule in disease[76].

1.2.2 SPHINGOLIPID PRODUCTION AND METABOLISM

Sphingosine is an 18-carbon amino alcohol with an unsaturated hydrocarbon chain that is one of the active metabolites of sphingolipid[83]. S1P is produced by the phosphorylation of sphingosine by kinase enzymes[84]. Sphingolipids biosynthesis starts from the cell membrane upon internal or external signal leading to the conversion of sphingomyelin into ceramide by sphingomyelin synthases[73,77]. Ceramide, one of the bioactive molecule in this pathway, is then metabolized to sphingosine by the enzyme ceramidase[74] (Figure 2).

In addition to its generation from sphingomyelin, ceramide can be synthesized *de novo* from serine and palmitate[74]. Sphingosine is phosphorylated by two sphingosine kinases to produce sphingosine-1-phosphoate (S1P)[85]. S1P is metabolized irreversibly by sphingosine-1-phosphate lyase, an enzyme localized in the endoplasmic reticulum ethanolamine and hexadecanol[86] or can be converted back to sphingosine by S1P phosphatase[87].

S1P produced inside the cell is then exported to the extracellular environment, so it functions in an autocrine and paracrine manner[88]. Due to its polar head group, S1P is unable to cross the cell membrane. Studies reported the involvement of ABC transporters in this process[89]. To a lesser extent, Spinster2 transporter was also suggested as a way of S1P export[89]. A concentration gradient of S1P exists between the tissue and plasma. Plasma levels are higher than tissue concentrations and total plasma concentrations being much higher than required to activate the receptors[90]. S1P has been found in numerous cells with plasma concentration ranges from 0.2 to 0.9 μM[75]. It appears to bind to plasma albumin and apolipoprotein M[90]. This binding lowers the concentration of free to S1P to the level closer to K_d value of the receptors[91]. S1P exerts its effect by binding to a particular family of G protein-coupled receptors (GPCRs), triggering certain intracellular and extracellular actions[76, 92].

As a determinant of cell fate, the three signaling molecules remain in a dynamic balance within the cell[93]. This balance is maintained by the SphK and S1P phosphatase which convert S1P back to sphingosine[76]. Ceramide and sphingosine are implicated in cell death and growth arrest and S1P is implicated in proliferation and survival. This balance is proposed to be the regulator of cell survival in response to stimuli[94, 95]

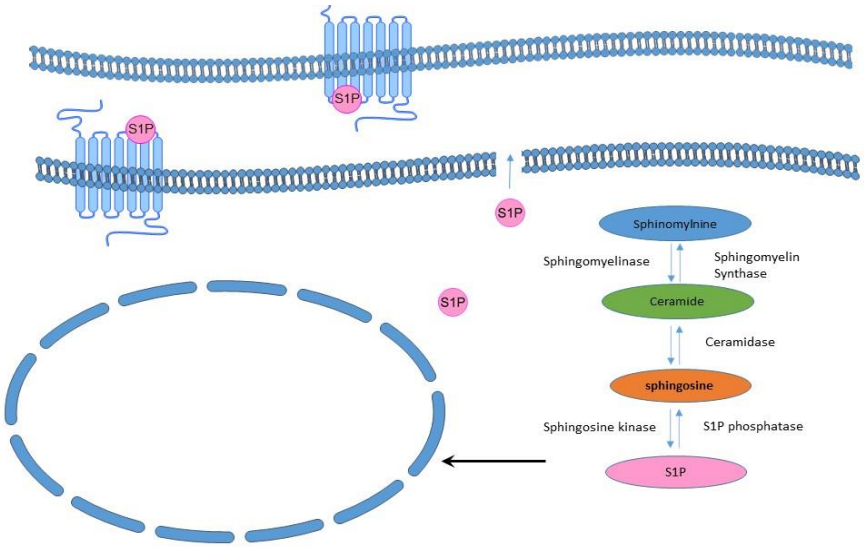

Figure 2: Production and Export of Sphingosines: biosynthesis pathway of sphinosine-1-phosphate (S1P) production. The process starts from cell membrane sphingomyelin conversion to ceramide by sphingomyelinase. Ceramide can then be converted to sphingosine which eventually is converted to S1P by kinase enzyme. S1P can travel outside the cell and bind to G protein coupled receptors.

1.2.3 SPHINGOSINE-1-PHOSPHATE (S1P)

S1P is a lipid mediator that is involved in many physiological and pathological conditions mediating signaling cascades involved in cell proliferation, angiogenesis, apoptosis, regulating proliferation and angiogenesis[89]. S1P functions as a dual messenger signaling molecule interacting with extracellular cell surface receptors and intracellular

receptor as a second messenger[96]. Extracellular S1P acts via interaction with G-protein-coupled receptors (S1PR1-5)[97-99]. Receptor studies revealed that cells express more than one S1P receptor type[100]. Upon certain stimuli, such as growth factor, cytokines or hormones, SphK starts the synthesis of S1P[101]. This production is also regulated by the intracellular levels of sphingolipids and sphingolipid metabolizing enzymes. SphK exists in two isoforms, SphK1, and SphK2[102]. They have varying tissue distribution, substrate specificity, and cellular localization. SphK1 is located in the cytosol and SphK2 is positioned in the nucleus[103].

1.2.3.1 EXTRACELLULAR FUNCTION OF S1P

S1P regulates several cellular functions through binding to G-protein-coupled receptors[85]. S1P binds to five GPCRs, S1PR1-5, leading to a series of downstream signaling pathways explaining the diversity of its function[100]. S1PRs, like other GPCRs, activates different heterotrimeric G proteins. Expression levels, binding affinity and pathway activation are the reasons for the wide variety of actions S1P has.

S1P regulates an ample range of cellular functions by activation of different G proteins. Pathway activation through coupling of S1P with its receptor varies depending on the receptor involved. The diversity of the signal generated is achieved by binding of the receptor to different alpha subunits in the G protein (Gs, Gi/o, Gq/11, and G12/13)[104]. This binding, results in various downstream cellular effects leading to activation of adenylyl cyclase inhibition as well as the Ras/MAP kinase cascade and (PI3 kinase)/Akt, phospholipase C (PLC), and small Rho GTPases[105].

S1PR1 couples only to Gi protein regulating different cellular functions including migration, proliferation, survival, cell–cell contact, angiogenesis, and lymphocyte trafficking[104]. At nanomolar concentrations, S1P activates extracellular signal-regulated kinase (ERK) and PI3K pathways[85]. This binding activates a series of the downstream signaling pathways and eventually lead to Akt activation and therefore induction of cell survival and Rac activation to induce cell migration[106]. Gi activation also leads to Ca^{2+} release from intracellular stores through activation of phospholipase C (PLC)[107]. Activation of PI3K pathway leads to a series of modulation of protein kinases Akt subsequently, leading to nitric oxide synthase (eNOS) activation. These series of signaling pathways produce a wide variety of biological functions such as angiogenesis, proliferation, and migration[108,109].

S1PR2 and S1PR3 couples to Gi, Gq, and G12/13 subunits[107]. Again, both receptors activate the Gi subunit leading PLC to induce IP3 formation and Ca^{2+} mobilization. However, they do this with different preferences[110]. S1PR2 activates Rho and inhibits Rac while S1PR3 activates both Rho and Rac[108]. S1PR2 regulates the anti-proliferation activity of S1P[111]. Moreover, S1PR2 inhibition of Rac was shown to inhibit migration[111]. Differences in the binding preference between S1PR2 and S1PR3 lead to a different biological response.

S1PR2 inhibits cell migration in normal and cancer cells via Rac inhibition[112]. Therefore, cells that express S1PR2 are highly susceptible to S1P treatment to prevent migration. Yamaguchi *et al*[113] reported inhibition of lung metastasis in melanoma cells expressing S1PR2 after treatment with S1P. S1P4 couples to Gi and G13 activating PLC and ERK. S1P5 couples to G12 and Gi[114]. Their binding to these subunits produce the same biological actions.

Tissue expression of S1PRs is another major determinant of its biological action. These receptors are expressed in different tissues. S1PR1, S1PR2, and S1PR3 are more

widely expressed receptors with S1PR1 being most abundant receptor among all S1PRs[115]. S1PR1 has high expression levels in the brain, cardiovascular system, kidney, and muscle [112]. S1PR2 shows broad tissue gene expression[109]. Animal studies with receptor knockout models have revealed the important biological function of S1PRs. The importance of S1PR1 for vascular maturation and its important role in angiogenesis is well reported. The S1PR1 null mice died in utero because of defective vasculature[116].

S1PR2 is involved in nervous system diseases[117]. Hearing loss and balance resulted from neuro-degradation in S1PR2 knockout mice[118] is an example of its key role the nervous system. Expression levels of S1PR4 and S1PR5 are generally less than the other three receptors. S1PR4 is restricted to lymphoid tissue and hematopoietic cells[109]. S1PR5 is expressed mainly in CNS, namely brain, white matter tracts, oligodendrocytes[75].

S1P anti-proliferative action was reported in different studies using hepatocytes. Ikeda *et al* [119] reported that S1P exerted antiproliferative action by binding to S1PR5 and activation of Rho. However, no further studies reported this effect. In another study, S1PR2 seemed to be responsible for the anti-proliferative effect of S1P.

Regulation of cell migration by S1P was reported in different cell lines. The migratory cellular response is also receptor dependent process. Overexpression of S1PR1or S1PR3 induces cell migration[120]. On the other hand, S1PR2 overexpression decreased cell migration via Rac inhibition[111]. Therefore, it is more likely that the action of S1P is determined by the level of receptor expression.

1.2.3.2 INTRACELLULAR FUNCTION OF S1P

In addition to its extracellular role, S1P can act on intracellular targets that are independent of its cell surface receptors. The exact mechanism of S1P intracellular action is still unknown; several studies report different targets for its intracellular action. S1P role in cell proliferation has been examined enormously. Promoting cell growth, survival, angiogenesis[85 116] and migration[121] are the main functions under investigation. These functions are associated with its extracellular G protein-coupled receptors. Regulation of S1P levels within the cell is an indicator for its intracellular action. However, these intracellular targets are not clearly elucidated.

The presence of intracellular S1P is indicative of its intracellular function. The intracellular action of S1P is believed to be the opposite of its extracellular action. In yeast, *Saccharomyces cerevisiaei*, exogenous S1P has no reported affect on cell growth[109]. Since yeast do not express cell surface receptors for S1P, its function might be solely intracellular. Accumulation of S1P in yeast resulted in growth suppression[109]. Maceyka *et al*. reported the opposing function of SphK enzymes[122], the two isozymes responsible for phosphorylation of sphingosine to give S1P[123]. While many studies reported the anti-apoptotic action of SphK1[115], SphK2 was observed to suppress growth and induce apoptosis[122].

S1P formed by SphK2 inhibits histone deacetylases[103]. S1P produced by SphK2 prevents the removal of acetyl groups within the histone by inhibiting the enzymatic activity of HDAC1 and HDAC2[124]. Therefore, HDAC appears to be an intracellular target for S1P. SphK2 is predominantly localized to the nucleus[125], and its action appeared to be the opposite of SphK1. SphK2 was reported to be associated with growth arrest and apoptosis, and this action depends on cell type[126]. Liu *et al.* reported that SphK2 increased doxorubicin-induced

cell death[127]. These reports indicate SphK2's different actions. SphK2 increases the release of cytochrome c into cytosol that initiate apoptosis by activation of caspase 3[127]. This apoptotic effect is receptor independent[128].

S1P was reported to induce apoptosis in different cell lines such as hMFs[129], MDA-MB321 breast cancer cell line [92], and B16 melanoma cells[128]. This effect was neither due to conversion of S1P to ceramide[129] nor to its effect through cell surface receptors[128]. Induction of apoptosis by SphK2 depends on Ca^{2+} mobilization from internal stores where S1P is believed to act as a second messenger[76]. Additional evidence supporting the intracellular function of S1P is the SphK2 inhibitor that suppressed the proliferation of MDA-MB-231 breast cancer cell line[130]. Although S1P was reported as an anti-apoptotic molecule, exogenous addition of S1P induced apoptosis that does not involve S1PR ligation, suggesting that S1P induced apoptosis is achieved by intracellular mechanisms[109]. S1PR1-3 knockout mice showed reductions in tumor size providing additional evidence in ruling out S1PR involvement in the process[131]. FTY720, a sphingosine analog, was reported to produce apoptosis by being phosphorylated with SphK2[132]. All previously mentioned evidence demonstrate that S1P has an intracellular action.

1.2.4 SPHINGOLIPIDS AND CANCER

Involvement of sphingolipid pathways in cancer has been reported in several studies. Alterations in sphingolipid metabolite levels and enzyme expression in cancer have strengthened the association between sphingolipid pathway and pathophysiology, progression and treatment resistance of the disease.

The role of ceramide has been studied extensively in cancer. Given its biological action as a proliferation inhibitor[109], many studies reported its effect on inducing apoptosis in tumors. On the other hand, S1P plays the opposite role inducing proliferation and increasing resistance to chemotherapy. Moreover, sphingolipid metabolizing enzymes, especially SphK, are upregulated in many tumors[80], consequently increasing tumor progression and metastasis. Plasma levels of S1P received ample attention in cancer. S1P levels were reported to be high in some tumors as a result of SphK1 overexpression[133]. S1P phosphatase and SPL are the two enzymes that regulate the concentration of S1P[86 134]. SPL overexpression was reported to enhance the action of cisplatin[135].

1.2.4.1 S1P RELATED GENE EXPRESSION IN CANCER

The SphK1 expression is correlated with increased tumorigenic potential, poor prognosis, and shorter disease survival. SphK1 up-regulation in a variety of human tumors suggests its action as an oncogene[102]. Elevated plasma S1P levels and decreased SPL, the main mechanism of S1P degradation, and SPP in expression levels in tumor tissue are inconsistent with the role of S1P in promoting tumor growth. Down-regulation of these enzymes provides supporting evidence for the role sphingolipids play in tumor progression[102].

1.2.4.2 S1P EFFECT ON CELL SURVIVAL

SphK (SphK1 and SphK2) produces S1P by phosphorylation of sphingosine. Although the two enzymes produce intracellular S1P, they are encoded by distinct genes and

have different biological functions[87]. The oncogenic activity of SPhK1 is related to its high mRNA expression levels in various tumors[136]. S1P binding to its G-protein coupled receptor (S1P1−5) initiates signal transduction. Several growth are affected by S1P signaling such as platelet-derived growth factor, epidermal growth factor (EGF), and vascular endothelial growth factor (VEGF)[137].

S1P as a biomarker for cancer detection was reported in a study conducted by Nunes *et al.* The study showed that circulating S1P levels were lower in patients with prostate cancer compared with healthy patients, which suggests S1P levels may reflect early metabolic changes in prostate cancer patients and represents an early marker for progression of tumor. Upregulation SphK1 and S1PRs were reported in prostate cancer cells[138].

1.2.4.3 S1P EFFECT ON CELL PROLIFERATION

Targeting the S1P pathway for cancer treatment is based on the fact that S1P contributes in tumorigenesis. The anti-apoptotic effect of S1P and mitogenic effect in most cell types is well recognized[78]. These effects may be dependent or independent on the S1P receptor as reported in S1P receptor deletion studies[139].

Autophagy triggered by S1P plays an important role in tumor cell survival. Metabolic stresses activated autophagy through S1P5 receptor and is documented in human prostate cancer cells. PDGF, VEGF, and TNF-α induces S1P production mediating tumor progression. The generated S1P interacts with S1P receptors on the cell surface of the same or a nearby cell and promotes tumorigenesis. This effect was further studied using prostate cancer cells

with high expression of SphK1[140]. Furthermore, SphK1 knockdown and SphK inhibition studies showed a reduction in tumor progression[102].

SphK1 is a critical component of EGFR signalling, which was demonstrated by Sarkar *et al*[141]. EGF is an important growth factor for MCF7 cell proliferation, progression, and invasion. This study showed the importance of SphK1 in EGFR signaling. The effect of SphK1 expression on EGF-induced proliferation was examined in MCF7 cell line where results indicate that overexpression of SphK1 protects the cell line from apoptosis and low levels reduced cell growth[141]. The results provide supporting evidence for SphK1's critical role for the growth and metastasis of human breast cancer. Similar results were observed in prostate cancer, bladder cancer, and melanoma[102]. These observations indicate that SphK1's role in mediating survival is common to numerous malignant cell types.

Decreased degradation of S1P by SPL has a similar effect to SphK1 overexpression. The exact role of SPL is not determined. However, several studies reported on the effect of low levels of SPL on decreasing apoptosis and sensitivity of chemotherapy drugs[102].

1.2.4.4 S1P EFFECT ON MIGRATION AND METASTASIS

Metastasis is one of the hallmarks of cancer. S1P affects cell invasion by the receptor-dependent mechanism. $S1P_1$, $S1P_3$, and $S1P_5$ activate cell migration while $S1P_2$ receptor may inhibit cell migration[142]. Cell migration is initiated by degradation of extracellular matrix-by-matrix metalloproteinase (MMPs). This enzyme is likely to be overexpressed by S1P[143].

S1P mediates cell invasion by different mechanisms. These mechanisms include enzyme regulation and cell adhesion. Glioblastoma invasiveness is mediated by S1P through

the activation of plasminogen activator inhibtor-1. On the other hand, anti-migratory effects of S1P were observed in glioblastoma exclusively expressing S1PR2. The anti-migratory effect was achieved through RhoA/Rho-kinase activation[102].

Melanoma expressing S1PR2 cells were reported to exhibit an anti-migratory and pro-migratory effect in the same cells expressing S1PR1[143]. Tumors expressing high levels of S1PR2 inhibited cell migration. S1PR3 expressing tumors have a positive affect on cell migration. These findings demonstrate the receptor dependent manner by which S1P mediates tumor cell migration and metastasis[102 142]. Ovarian cancer cell invasion was activated by S1P through activation of ERK and AKT pathways. Enhanced tumor cell migration was observed in pancreatic cancer with high expression levels of SphK1. Similar results were reported in breast cancer cells[143].

1.2.4.5 SIP EFFECT ON ANGIOGENESIS

Tumor growth and metastasis are governed by angiogenesis. Angiogenesis is a crucial step in solid tumor metastasis and progression. Angiogenesis was stimulated by S1P production *in-vivo* and *in-vitro*[107]. The role of S1P in angiogenesis has been reported in several studies. Angiogenic and tumorigenic effects of S1P was tested *in-vivo* and *in-vitro*.

S1P induces angiogenesis by up-regulating proangiogenic growth factors, basic fibroblastic growth factor (bFGF) and vascular endothelial growth factor (VEGF) as well as cytokines implicated in angiogenesis[75 144]. VEGF was reported to be up-regulated by S1P in prostate cancer cells[140]. VEGF induces S1P receptor expression in bovine aortic endothelial cells BAEC[140 145]. S1P and VEGF have a synergistic effect on angiogenesis in inducing S1PR1[145]. Inhibition of SphK1 decreased VEGF-induced expression of adhesion molecules.

Inhibition of TNFα induced apoptosis in endothelial cells by S1P provides supporting evidence for its role in blood vessels proliferation[143].

S1PR1 siRNA has been reported to decrease vascular stabilization, angiogenesis and tumor growth *in-vivo*[102]. Expression of S1PR1 is up-regulated in the tumor vasculature during angiogenesis. Chae *et al.* described that down-regulation of S1PR1 expression with siRNA inhibited angiogenesis and tumor growth *in-vivo*. This result is in agreement with the role of S1P as a pro-angiogenic factor[146]. High expression levels of S1PRs in vascular endothelial cell are well known where it was originally identified, therefore, named Edg (endothelial differentiation gene). Lack of S1PR1 showed impaired angiogenesis in mice.[116] Additionally, impaired angiogenesis by antisense S1PR1 oligonucleotides demonstrates the crucial role of S1P signaling in endothelial cells proliferation and migration[147].

Activation of S1P receptors increases production growth and pro-angiogenic factors such as VEGF implicating of S1P in cancer progression[148]. A study conducted by Watson *et al.* showed that increased S1PR expression level is associated with shorter time to recurrence and associated with shorter disease-specific survival times. These results provide supporting evidence for the role S1P plays in tumor progression[149].

Formation of new vessels by remolding of extracellular matrix (ECM) is important for angiogenesis. Matrix metalloproteinase (MMPs) are involved in matrix remodeling enabling formation of new blood vessels. MT1-MMP is activated by S1P and has been shown to promote the formation of new blood vessel rat. MT1-MMP–knocked out mice showed no angiogenic response to fibroblast growth factor 2[150 151]. Lowering S1P levels using anti-S1P monoclonal antibodies inhibited angiogenesis induced by VEGF and bFGF in murine xenograft and allograft models[144].

The crucial role of S1P signaling in tumor angiogenesis is consistent with the progressive nature of tumor $S1P_1$ expression which is strongly induced in tumor vessels as blockade of this pathway inhibited tumor angiogenesis.

1.2.5 SPHINGOLIPIDS AND BREAST CANCER

Overexpression of SphK1 has been reported in stomach, lung, brain, colon[152], kidney, and breast cancer[87]. (ER-) Breast cancer was found have higher expression levels of SpkK1 than (ER+) suggesting that increased SphK1 expression has a strong association with shorter disease-free and disease-specific survival.

Increased ceramide and sphingosine levels have been described in MCF-7 breast cancer cell line after treatment with doxorubicin leading to cell apoptosis. Overexpression of SphK1 in MCF-7 cells strengthens the involvement of S1P in the progression of breast cancer subtypes. Increased SphK1 expression is associated with acquisition of resistance to tamoxifen leading to reduced patient survival[149]. S1P produced inside the cells is transported to the hostile extracellular environment of cancer cells. Binding of S1P to its receptors promotes proliferation of cancers cells as previously discussed. S1P is known to exert its intracellular functions, but its relevance to cancer biology is not well known[98].

SphK1 may promote breast, growth, and responsiveness to estrogen. The tumorigenic effect that SphK1 has in breast cancer cells was found to be associated with its overexpression[153]. SphK1 activation by estrogen signaling promotes estrogen-dependent oncogenesis[154]. Datta et al correlated high expression levels of SPHK1 in TNBC cells with

poor overall and progression-free survival. The study also correlated elevated SphK1 with poor response to Doxorubicin treatment[155]. These results were demonstrated by SphK1 inhibition in TNBC cell lines using SKI-5C, a pharmacological inhibitor of SphK1, which in turn sensitized TNBC cells to doxorubicin chemotherapy[155]. Increased SphK1 expression in a variety of solid tumors brought attention to the role sphingosine plays in cancer[156]. *In-vitro* studies revealed the involvement of sphingosines in cancer cell proliferation, survival, and migration[76].

The role sphingosines have in cancer has been investigated in different studies. Cellular processes namely, apoptosis, proliferation, angiogenesis metastasis are regulated by sphingolipids in cancer. In addition to their role as a second messenger, sphingolipids alter cellular metabolism in normal cells. Sphingolipids metabolites, including sphingosine, ceramide, and S1P, provide structural and functional support to growing cells[98].

An impaired apoptotic mechanism is a hallmark of tumor cells. Normal cells initiate apoptosis to terminate abnormalities. Sphingolipids metabolites have proapoptotic as well as anti-apoptotic properties. The balance between survival and death is termed rheostat model where ceramide and sphingosine, the proapoptotic molecules, levels are elevated in response to various stimuli to initiate programmed cell death. S1P is the anti-apoptotic and initiates cell signals to promote cell growth and proliferation[74,75, 92].

1.2.5.1 SPHINGOLIPIDS AND TNBC/BLBC

Triple negative breast cancer is associated with poor prognosis, tumor aggressiveness, and limited therapeutic options. SphK1 expression is correlated with poor prognosis that can

be attributed to increased levels of S1P[157] leading to increased migration and chemoresistance[87].

SphK1 expression was found to be higher in many human tumors than in normal tissues[78]. Studies revealed that sphingosine kinase enzyme SphK1 has been associated with tumor lymphangiogenesis[158]. On the other hand, few studies indicate the opposite action of SphK2. Antoon *et al*. reported that SphK2 inhibition induced intrinsic apoptosis in chemoresistant breast cancer[159]. Triple negative MDA-MB-231 breast cancer showed high expression levels of SphK1[159].

Previous findings indicate both SphK1 and SphK2 are involved in TNBC progression; however, which isozymes plays more significant role in tumor growth needs further investigation. Differences in expression levels of SphK1 or SphK2 can change sphingolipid levels and cause pro- or anti-cancer behaviors. However, roles of sphingolipid plays in triple negative breast cancers are not well studied compared to ER-positive cancers, therefore whether S1PRs are involved and which receptors affect tumor progression remains unclear. Different breast cancer subtypes respond differently to the sphingolipid pathway. These differences can be utilized to determine the prognosis of the disease. Understanding the mechanism of S1P involvement in breast cancer would expect to lead to new therapeutic strategies.

1.2.6 S1P AND CHEMORESISTANCE

Cancer cells adapt various mechanisms to protect themselves from the harmful effect of anti-cancer drugs. As previously mentioned, ceramide–sphingosine–S1P rheostat is an important modulator of cell fate with ceramide and sphingosine being apoptotic molecules

whereas S1P is considered as anti-apoptotic molecule[75]. According to the model, adjusting this balance between ceramide and S1P can increase or decrease resistance to chemotherapy[82]. Given the significant role sphingolipids have on cell survival, altering the metabolism of sphingolipids by overexpressed xenobiotic metabolizing enzymes can increase cancer cell resistance to chemotherapy.

Many anticancer drugs increase ceramide accumulation leading to apoptosis and cell death[160]. Apoptosis occurs due to activation of caspases and loss of cytochrome c[161], and increased degradation of ceramide and inhibited generation is adapted to suppress apoptosis[162].

Ceramide/S1P rheostat implication in chemoresistance is correlated to SphK1 expression. Overexpression of SphK1 alters ceramide/S1P ratio causing more production of the oncogenic S1P in Panc-1 cells[163]. Consequently, Panc-1 cells become more resistant to gemcitabine treatment.

Higher S1P/ceramide ratio is associated with increased resistance to chemotherapy in melanoma cells. This is consistent with S1P being anti-apoptotic in tumor cells[164]. The overexpression of SphK1 in melanoma cells favors increased production of S1P, which implies decreased ceramide levels. Consequently, the contributions of dysfunctional sphingolipids metabolism in drug resistance have been studied in various human cancer cells. Dysregulation of sphingolipids metabolism by S1P overproduction inhibited apoptosis in HL-60 leukemia cell lines by doxorubicin. Furthermore, increased S1P degradation was associated with decreased resistance to cisplatin[165].

Multidrug resistance (MDR) in cancer chemotherapy has been associated with sphingolipid signaling pathway. S1P regulation of P-gp function was demonstrated in rat

brain endothelial cell line RBE4[166]. SphK1 overexpression in RBE4 cells induced P-gp expression suggesting the crucial role S1P plays in the development of the MDR. The effect of S1P on P-gp transport activity through $S1P_1$ and $S1P_3$ receptors was determined by measuring vinblastine accumulation of inside RBE4 cells. Increased vinblastine efflux was attributed to increased P-gp activity mediated by SphK1 overexpression[166]. Additionally, HL-60 cell, an acute myeloid leukemia cell, with high expression levels of SphK1 showed increased resistance to chemotherapeutic drugs namely, doxorubicin and etopside[167]. This finding provides supporting evidence of S1P implication in MDR in cancer. Inhibition of apoptosis induced by anticancer drugs due to SphK1 overexpression in tumor cells led to the development of SphK1 inhibitors to overcome chemoresistance. The use of these inhibitors demonstrated increased sensitivity to chemotherapeutic drugs by inhibiting S1P synthesis[143]. These findings support the role S1P plays MDR proteins expression subsequently in cancer progression and chemoresistance. High S1P receptor expression levels, S1PR1, and S1PR3, are associated with resistant to tamoxifen treatment[149].

1.2.7 TARGETING S1P PATHWAY IN CANCER THERAPY

As mentioned above, the S1P pathway has been implicated in cancer pathogenesis. Strategies for targeting S1P production and function in cancer is promising. Current strategies for targeting S1P signaling in cancer focus mainly on modulating S1P receptor signaling and/or SphK inhibitors. Blocking S1P production in tumor cells and or altering its level is the basic concept of these strategies. The combination of chemotherapeutic agents with sphingolipid pathway modulators to achieve a synergistic effect and more selective targeting is being investigated.

Modulating S1P receptor signaling by FTY720, an S1P analog which binds to four of five S1P receptors ($S1P_1$, $S1P_3$, $S1P_4$, $S1P_5$), was found to induces apoptosis *in-vitro* in several cancer cell lines[168]. Furthermore, FTY720 stopped tumor growth and metastasis in a mouse model of breast cancer. Cancer cells showed high susceptibility to treatment with FTY720[168]. Although FTY720 is a S1P receptor agonist, it was found to reduce angiogenesis and vascularization in tumors suggesting a new approach for treatment[169].

Another approach for targeting S1P is depletion of extracellular S1P by S1P specific antibodies. LT1009 reduced proliferation and increased apoptosis in tumor growth and metastasis in breast and ovarian cancers[170]. This approach was based upon the abundant levels of S1P in the extracellular environment that is above the K_d of S1P receptors and its reported effect on proliferation and metastasis.

SphK1 overexpression in a variety of solid tumors makes it a rational target for selective treatment. In general, the main target for these inhibitors is to block or decrease the production of S1P and increase the concentration of ceramide. Inhibition of SphK1 *in-vitro* and its effect on S1P production has been extensively studied using dimethyl sphingosine, an N-methylated metabolite of sphingosine. The inhibition was observed on the growth and metastasis of melanoma cancer cells[102]. Another SphK inhibitor, SK1-I (BML-248), induced apoptosis in leukemia cells[171].

1.2.7.1 S1P AS AN ANTICANCER AGENT

The production a synergistic effect using drug combination is a common strategy for cancer therapy. The aim of combination therapy is to produce maximum kill and avoid drug resistance. In an attempt to find more useful drug combination for treating different tumors, a few *in- vitro* studies reported the beneficial use of S1P in combination with various clinically available chemotherapeutic drugs. Dr. Yang's research group reported that exogenously

administered S1P exhibited synergistic effects with chemotherapy drugs doxorubicin and docetaxel in human breast cancer MCF7 and MDA-MB-361 cells[92 172].

Their research utilized S1P in combination based on the fact that S1P itself can produce cell death. This combination maybe the next era of cancer treatment.

2. OBJECTIVES AND HYPOTHESIS

2.1 OBJECTIVES:

1. Understanding whether S1P can selectively induce apoptosis in human TNBC/BLBC cell lines at low µM concentration range.
2. Investigating whether S1P can exert a synergistic and/or additive effect with chemotherapeutic agents doxorubicin and docetaxel towards TNBC/BLBC.

2.2 HYPOTHESES:

We propose sphingosine-1-Phosphate (S1P) as a target for breast cancer treatment.

1. S1P induces apoptosis and necrosis in breast cancer cells at a concentration above 0.1 µM.
2. S1P sensitizes human breast cancer cell to chemotherapeutic agents.

3. MATERIALS AND METHODS

3.1MATERIALS

3.1.1 CHEMICALS AND CELLS

Sphingosine-1-phospahate (S9666- 1 mg), docetaxel (01885- 5 mg), and doxorubicin (D1515- 10 mg) were purchased from Sigma-Aldrich (Oakville, ON, Canada). Triple negative/Basal-like cell lines were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). Nine triple-negative breast tumor cell lines share a basal-like morphology (HCC1599, HCC1937, HCC1143, MDA-MB-468, HCC38, HCC70, HCC1806, HCC1187, DU4475).

Table 1: Summary of Triple Negative Breast Cancer Panel 1 (ATCC® No. TCP-1001™) illustrating cell line grade, histology, and mutation data.

Cell Line	Grade	Histology	Mutant Gene
HCC1599	TNM stage IIIA, grade 3	Primary ductal carcinoma	TP53,KDM6A
HCC1937	TNM stage IIB, grade 3	Primary ductal carcinoma	TP53, BRCA2
HCC1143	TNM stage IIA, grade 3	Primary ductal carcinoma	TP53,BRCA1
MDA-MB-468	NA	Adenocarcinoma	PTEN,TP53,SMAD4,RB1
HCC38	TNM stage IIB, grade 3	Primary ductal carcinoma	TP53,CDKN2A
HCC70	TNM stage IIIA, grade 3	Primary ductal carcinoma	PTEN,TP53
HCC1806	TNM stage IIB, grade 2	squamous cell carcinoma	CDKN2A,KDM6A,STK11,TP53
HCC1187	TNM stage IIA, grade 3	Primary ductal carcinoma	TP53
DU4475	NA	Carcinoma	APC, BRAF,MAP2K4, RB1

Abbreviations: **TP53**: tumor suppressor p53, **KDM6A**: Lysine (K)-Specific Demethylase 6**, BRCA1**: Breast Cancer 1 Tumor Suppressor, **BRCA2**: Breast Cancer 2 Tumor Suppressor, **PTEN**: Phosphatase and tensin homolog, **SMAD4**: SMAD Family Member 4, RB1: Retinoblastoma-Associated Protein, **CDKN2A**: Cyclin-Dependent Kinase Inhibitor 2A, **STK11**: Serine/Threonine Kinase 11, **MAP2K4**: Mitogen-Activated Protein Kinase 4. **BRAF**: B-Raf Proto-Oncogene, Serine/Threonine Kinase, **APC**: Adenomatosis Polyposis Coli Tumor Suppressor

Penicillin/streptomycin solution was purchased from Sigma Life Science (Kansas City, MO, USA). Leibovitz's L-15 medium was purchased from Life Technology (Carlsbad, CA, USA). RPMI-1640 medium was purchased from Hyclone (Logan, Utah), and Fetal Bovine Serum (FBS) was purchased from Sigma life Science. 0.25 % Trypsin-EDTA was purchased from Sigma Life Science. CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay, Cell CytoTox96®Non-Radioactive Cytotoxicity Assay System, and CellTox™ Green Cytotoxicity Assay were purchased from Promega (Madison, WI, USA). Annexin V-FITC Apoptosis Detection Kit was used to detect apoptosis. Kits were purchased from Biotool, Houston, TX. 96-Well Optical-Bottom Plates, 96 Well Plate Polystyrene, and 6 well plates were purchased from Thermo Scientific. Corning® 75cm² straight Neck Cell Culture Flask with Vent Cap (Corning, NY) and Thermo 25 cm^2 (Waltham, Massachusetts) were used for cell culture.

3.1.2 COMPOUNDS AND BUFFERS PREPARATION

Stock solutions were prepared for S1P dissolved in methanol at 2.64 mM, docetaxel in ethanol at 2 mg/mL, doxorubicin in water at 1 mM, 1x PBS buffer (pH 7.4) was prepared by dissolving the following chemicals in 800 mL distilled water: 8 g NaCl, 0.2 g KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4. The pH was adjusted to 7.4 by adding HCl and then the volume was adjusted to 1L. The final PBS solution was sterilized by autoclaving. 4% paraformaldehyde was prepared by dissolving 4g paraformaldehyde powder in 100 mL of 1x PBS, and the final solution was heated at 37°C water bath for 1-2 hours.

3.2 METHODS

3.2.1 CELL CULTURE

All breast cancer cell lines except HCC1599 were cultured in T-75 culture flasks according to ATCC protocol. The medium was supplemented with 10% FBS and 1% penicillin/streptomycin. HCC1599 breast cancer cell line was cultured in T-25. RPMI-1640 medium was used to culture all cell lines except MDA-MB-468, which was cultured using Leibovitz's L-15 medium. All cell lines were incubated under a humidified 5% CO_2 atmosphere at 37°C except cell line MDA-MB-468 was incubated under 0% CO_2 atmosphere at 37°C. Cell culture media were changed 2-3 times a week. Cells were sub-cultured by using 0.25% trypsin-EDTA solution after they reached 70 % confluence.

3.2.2 CYTOTOXICITY STUDIES

Cells for each were plated in 96-well plates at different density. The number of cells varied from each cell line. The plated cell number was 10000 cells/well for HCC1599, 15000 cells/well for HCC1937 15000/well, 10000 cells/ well for HCC1143, 7000 cells/well for MDA-MB-468, 7000 cell/well for HCC38, 7000 cell/well for HCC70, 7000cell/well, for HCC1806, 10000 cells/well for HCC1187, 10000 cells /well for DU4475. Cell number for each cell line was obtained for pilot studies. The cells in suspension were mixed with 20µl of CellTox™ Green dye and plated. All cells were incubated for 24 hours before being treated with S1P concentrations ranging from 20µM to 0.1µM. Cells were treated with S1P and left for an hour before applying the chemotherapeutic drug. Maximum LDH release buffer was used as positive control. Solvent only cell were used as negative control S1P cytotoxicity was measured at exposure time of 24h, 48h and & 72h using CellTox™ Green Cytotoxicity Assay kit according to manufacturer's instructions. The kit measures changes in membrane integrity

that occur as a result of cell death by staining DNA molecules of the dead cells. Fluorescence was determined at 485–500 nm Excitation/520–530nm Emission using a BioteK plate reader. Positive controls (Lysis buffer) and negative controls (solvents) were used and % cytotoxicity was determined using the following formula:

$$\%\ Cytotoxicity = \frac{Experiment - Negative\ Control}{Maximum\ Death - Negative\ Control} X100$$

The data were plotted as % cytotoxicity vs. S1P concentration. GraphPad Prism software was used to evaluate the data. The results were plotted as concentration vs % cytotoxicity at each time point.

3.2.3 CELL PROLIFERATION ASSAY

96-well tissue culture plates were seeded with different cell densities and incubated at 37ºC, and 5% CO_2 except for cell line MDA-MB-468 which was incubated at 0% CO_2 until they reached 70% confluence. Cells were then treated with S1P (concentration range: 0.1- 20 μM) and incubated for 72 h. Methanol treated cells were used as negative control. The assay was carried out by using the CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay kit, as per manufacturer's protocol. Plates were measured using a BioteK plate reader to measure the absorbance at 490 nm.

The number of living cells was measured by the quantifying formazan as a result of the MTS conversion by dehydrogenase enzyme in metabolically active cells (i.e. 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)). Data

were plotted as % increase of cell proliferation compared to the control vs. S1P concentration and the results were analyzed using GraphPad Prism software. The final data were plotted as % increase of cell proliferation vs. time in hours.

3.2.4 APOPTOSIS STUDIES

Annexin V-FITC kits were used for apoptosis detection. Annexin V-FITC kit detects the externalization of phosphatidylserine in apoptotic cells. Annexin V conjugated to green-fluorescent fluorescein isothiocyanate (FITC) dye binds to phosphatidylserine sites on the member of apoptotic cells. Propidium iodide (PI) binds to necrotic cells. Treatment of cell with Annexin and PI dyes stains dead cell but not live cells. Apoptotic cells give green fluorescence, and necrotic cells show green and red fluorescence.

Cells were seeded in six-well plates at a density of 10000 cells/well with two mL growth medium. After 24 h, the medium was changed to the adherent cell medium. Moreover, cells were treated with S1P alone at concentration obtained from the cytotoxicity studies for each cell line and methanol was used as a negative control. Nonadherent cells were treated after 4 h of plating. After 72 hr treatment, the medium and cells were collected in 15 mL conical tubes. 500-µL trypsin was then added to each well and after 2 min, the trypsin was neutralized by adding 200 µL of cell culture media containing FBS buffer. The cells were then collected and added to the previously collected medium. Collected cells were then centrifuged at 300g for 5 min at room temperature. Then, the supernatant was removed, and the plate was washed with PBS buffer. Cells were centrifuged again for 5 minutes. The supernatant was removed, and cells were suspended in 100µL buffer. 5 µL Annexin V -FITC and 5 µL PI Staining Solution were added to each 100 µL of cell suspension. Cells were then incubated for 15 min at room temperature. After incubation, 400 µL of 1 × binding buffer

was added to each tube and then transferred to flow cytometry tubes and kept on ice. Measuring the fluorescence emission was done using FACSCalibur™.

ZOE™ Fluorescent Cell Imager was used to capture an image of wells treated with S1P and non-treated wells. Images were taken after 72 h of treatment for both treated and non-treated cells.

3.2.5 CHEMOTHERAPY COMBINATION STUDIES

All nine cell lines were seeded in 96 well plates with varying density as described in the cytotoxicity studies. Cells were treated with different concentrations of chemotherapeutic drugs alone or in combination with S1P. Three different concentrations of S1P 10µM, 1µM,and 0.1µM. The two chemotherapeutic agents were selected to perform this part are docetaxel (concentration 5-40 µM) and doxorubicin (concentration 2.5-20 µM). After treatments had been administered, cell lines were incubated for 72 h at 37°C and 5% CO_2 except for cell line MDA-MB-468, which was incubated in 0% CO_2. Measurement of cell death was done using the CytoTox 96® Non-Radioactive Cytotoxicity Assay kit. This test is a colorimetric test that measures the conversion of a tetrazolium salt into formazan. This process is achieved by lactate dehydrogenase enzyme that is released upon cell lysis. The intensity of the color is proportional to the number of dead cells.

The BioteK plate reader was used to measure the absorbance at 490nm. The percent of cell death was determined by using the following formula:

$$\%\ Cytotoxicity = \frac{Experiment - Negative\ Control}{Maximum\ LDH\ Release - Negative\ Control} X100$$

The data obtained were grouped based on the type of treatment (Chemotherapeutic agent plus S1P and Chemotherapeutic agent alone). Each treatment was grouped into four group representing the four concentration of the chemotherapeutic drug used.

3.2.6 STATISTICAL ANALYSIS

GraphPad Prism software was for data analysis. The mean ± SD for each assay was calculated from three independent experiments with three replicate per each independent experiment. Comparison between the effects of the anti-cancer drugs alone or in combination with S1P was performed using the t-test. P-value <0.05 was considered significant.

4. RESULTS

4.1 CYTOTOXICITY STUDIES

The cytotoxic effect of S1P was evaluated for up to 72h. Each cell line was evaluated for exposure to a wide range of S1P concentrations (0.125-20µM) at 24, 48, and &72h. The response of the nine cell lines towards the S1P treatment of different concentrations was summarized in Table2 and Figure 3. S1P showed a time-dependent effect in the TNBC/BLBC cell lines.

In general, prolonged exposure to lower concentrations of S1P caused higher cytotoxicity compared to higher concentrations and highest toxicity was achieved after 72 hr of exposure.

HCC1937 (CRL2336) showed highest cytotoxicity among the nine cell lines after prolonged exposure to lower concentrations of S1P. Highest toxicity was achieved at 72 h exposure. Highest cell death of about 76 % achieved at 0.1 µM while the number of cell death decreases as S1P concentration was increased. S1P did not cause any significant increases in cell death in HCC1806 (CRL2335). Maximum cell death reported about 8% after prolonged treatment for 72h. The concentration that produced maximum cell death varied in cell lines. HCC1143 (CRL2321) showed increased cell death at 10µM. HCC70 (CRL2315) showed maximum cell death at 5µM.

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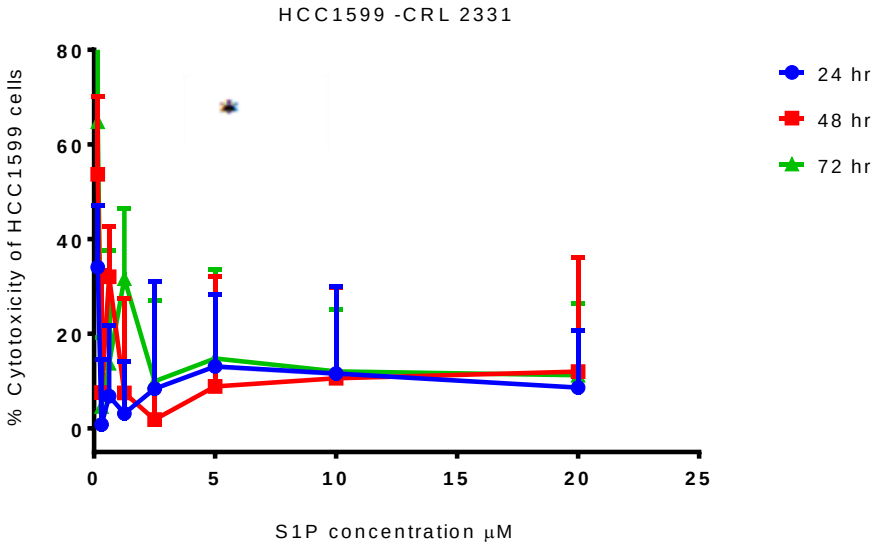
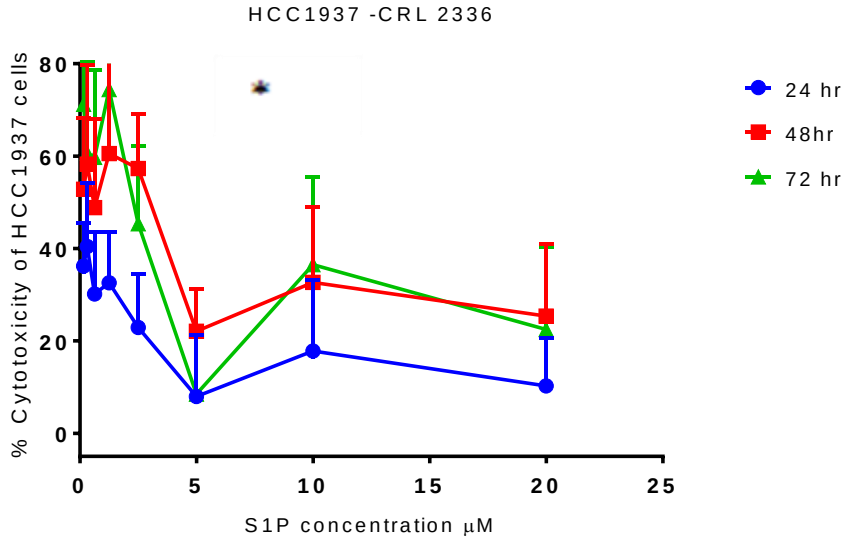
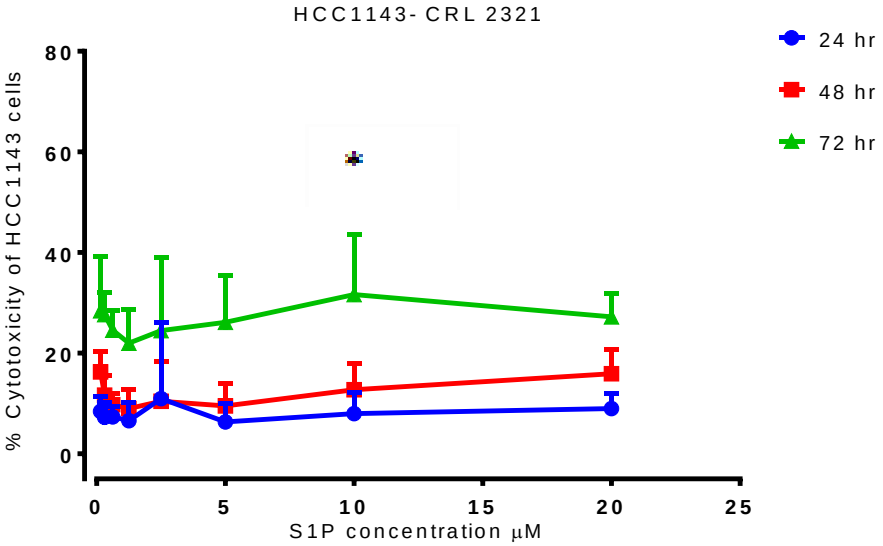

HCC1599 -CRL 2331
% Cytotoxicity of HCC1599 cells
S1P concentration µM
24 hr
48 hr
72 hr


HCC1937 -CRL 2336
% Cytotoxicity of HCC1937 cells
S1P concentration µM
24 hr
48hr
72 hr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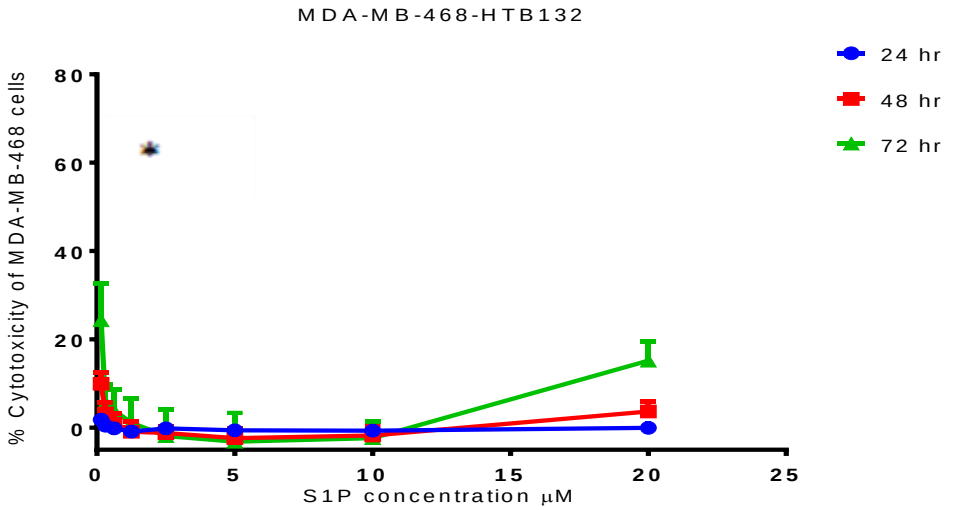
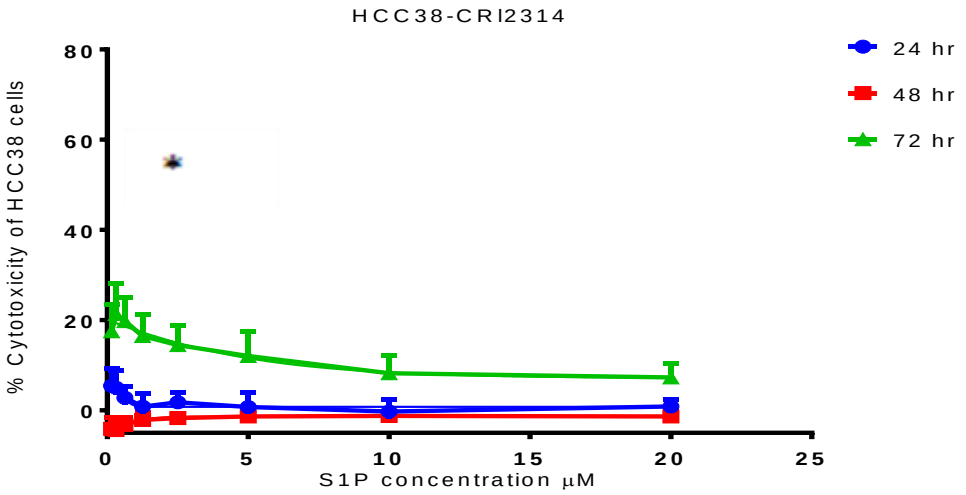
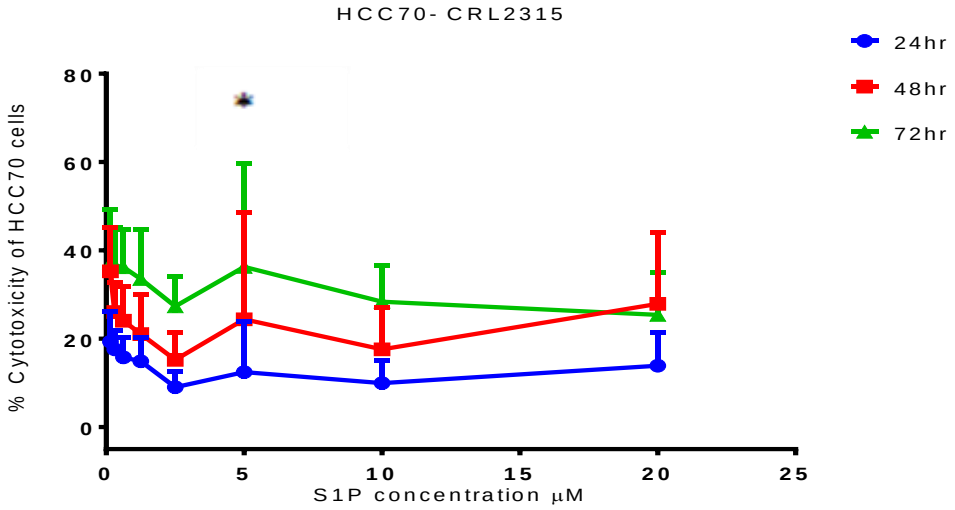
% Cytotoxicity of HCC1143 cells
S1P concentration µM
24 hr
48 hr
72 hr


MDA-MB-468-HTB132
% Cytotoxicity of MDA-MB-468 cells
S1P concentration µM
24 hr
48 hr
72 hr


HCC38-CRI2314
% Cytotoxicity of HCC38 cells
S1P concentration µM
24 hr
48 hr
72 hr


HCC70- CRL2315
% Cytotoxicity of HCC70 cells
S1P concentration µM
24hr
48hr
72hr

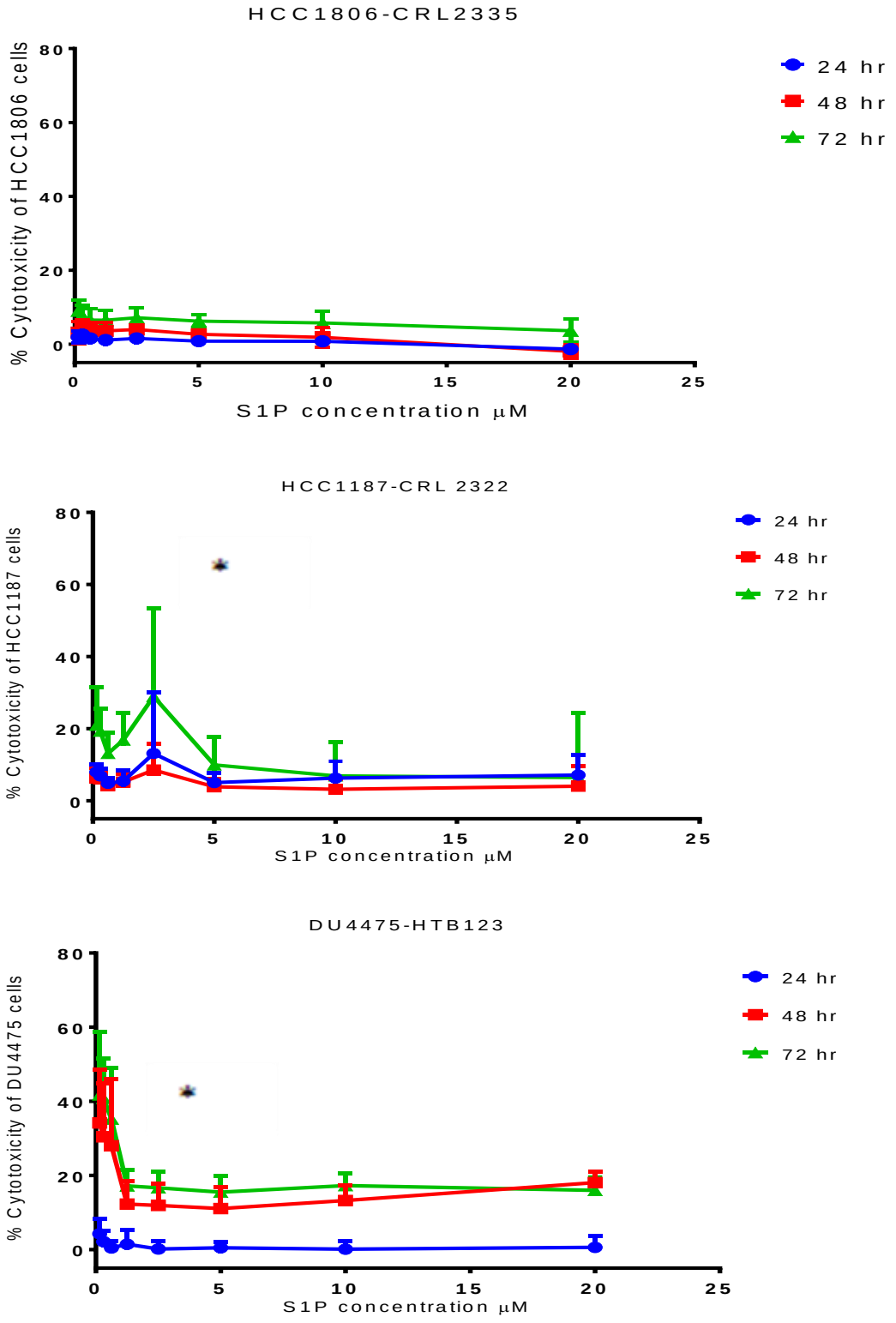


Figure3 : Percent cytotoxicity (mean±SD) of nine TNBC/BLBC cell lines following treatment with different concentrations of S1P for 24, 48 and 72 hr. Cells treated with complete growth medium and solvents were used as a control. The mean± SD was calculated from three independent experiments, *=P<0.05.

4.2 CELL PROLIFERATION STUDIES:

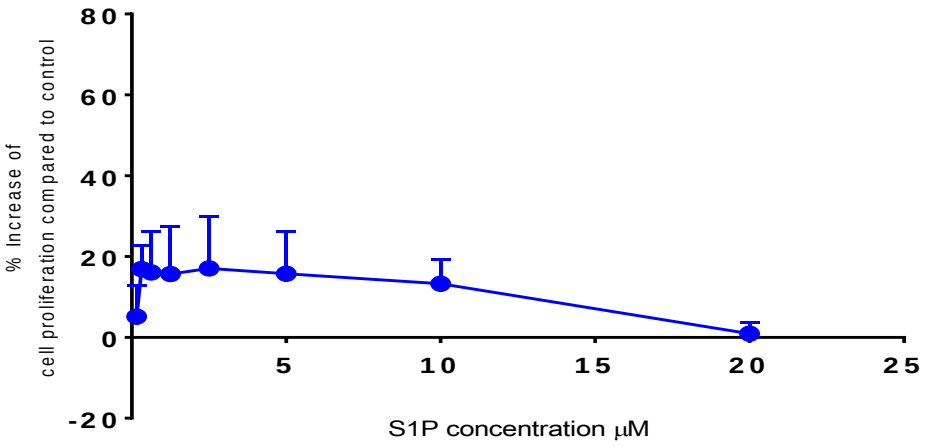
Cell proliferation was assessed using CellTiter 96 AQueous proliferation assay. The absorbance of MTS product, the metabolic conversion of a tetrazolium compound MTS to a colored product by live cell was measured. S1P showed a bell-shaped dose response curve on human TNBC/BLBC cell lines. All cell lines were exposed to different concentrations of S1P for 72 h.

HCC1599, HCC1937, HCC1143, MDA-MB-468, HCC38, HCC70, HCC1187, and DU4475 demonsterated a proliferation response while in cell line HCC1806, no response was observed. Maximal proliferation was achieved at 0.3 μM and 5 μM in responsive cell lines. Prolonged cell exposure to S1P concentrations of 10μM or higher inhibited cell proliferation.

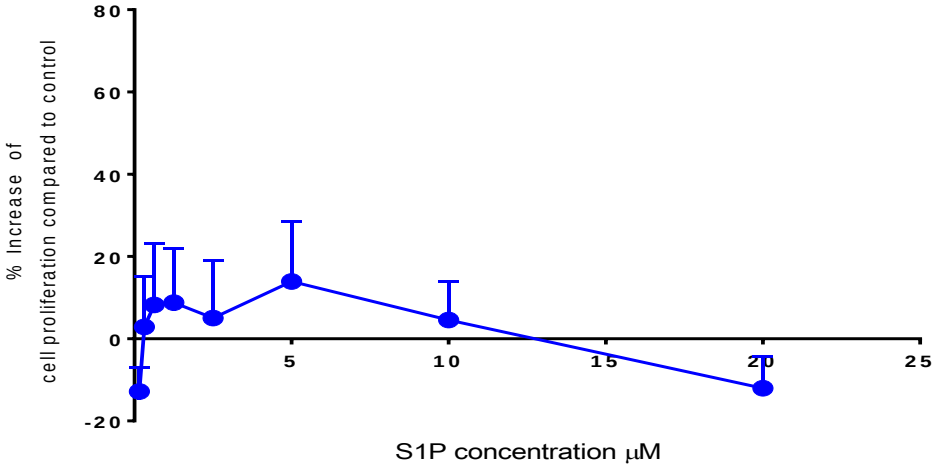
No inhibition of cell growth was observed in HCC38 cell line. HCC1187 cell line showed maximum proliferation at 2.5μM of about 12%. HCC1599 cell line was observed to have a higher percent of cell proliferation around 1% at 2.5μM. S1P did not produce any effect on cell proliferation for the HCC1806 cell line. HCC1937 cell line showed cell proliferation of about 17% at 2.5 μM. DU4475 cell line had plateaued proliferation of around 14% at 0.3-5μM. MDA-MB-468 also showed a plateau of proliferation around 14% at same concentrations. HCC1143 was observed to have a maximum proliferation of 16% at 2.5 μM. HCC70 cells reached high proliferation level of 7% at a concentration around 2.5μM. At higher S1P concentration the proliferation decreased.

In summary, S1P induced marginal proliferation in TNBC/BLBC cell lines with maximal effect of 16% increase over control at 2.5μN S1P towards HCC1599 cells.

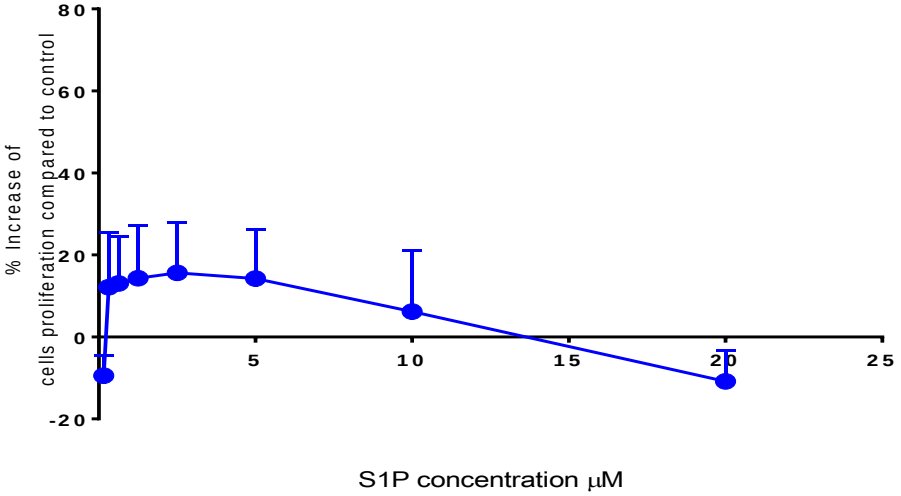
HCC1599 -CRL 2331

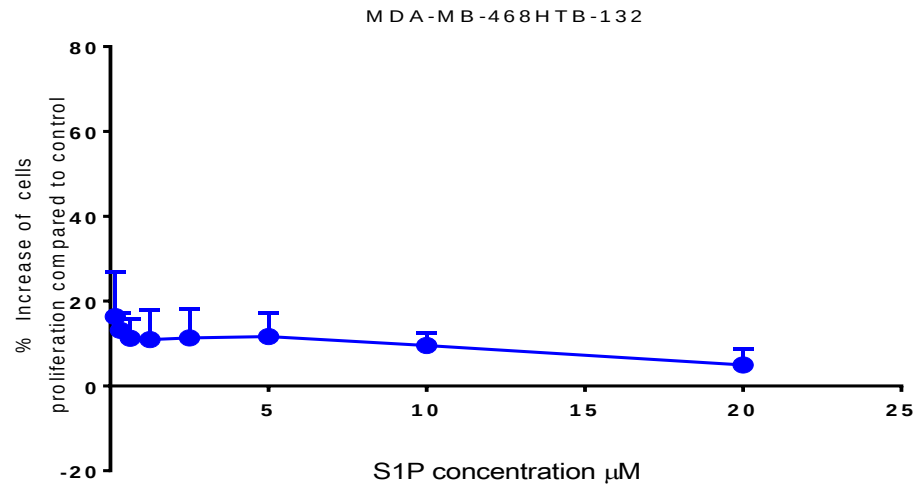


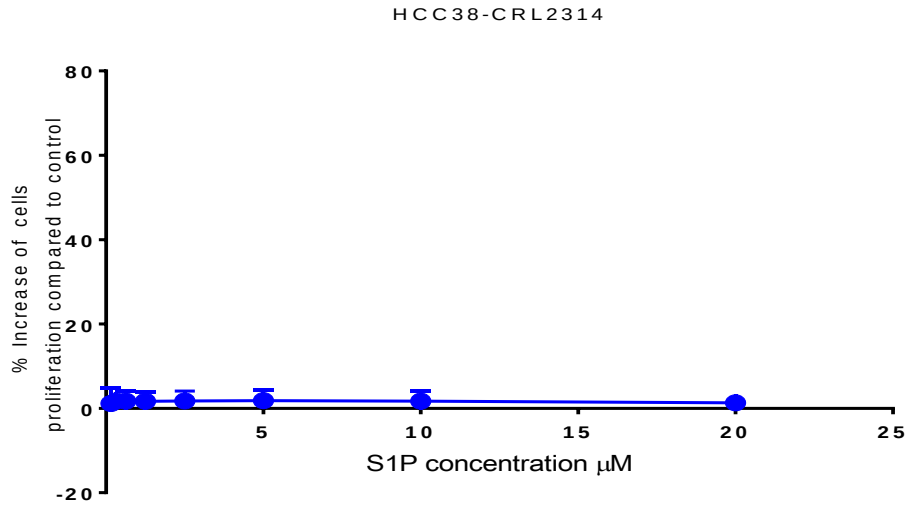
HCC1937 -CRL 2336

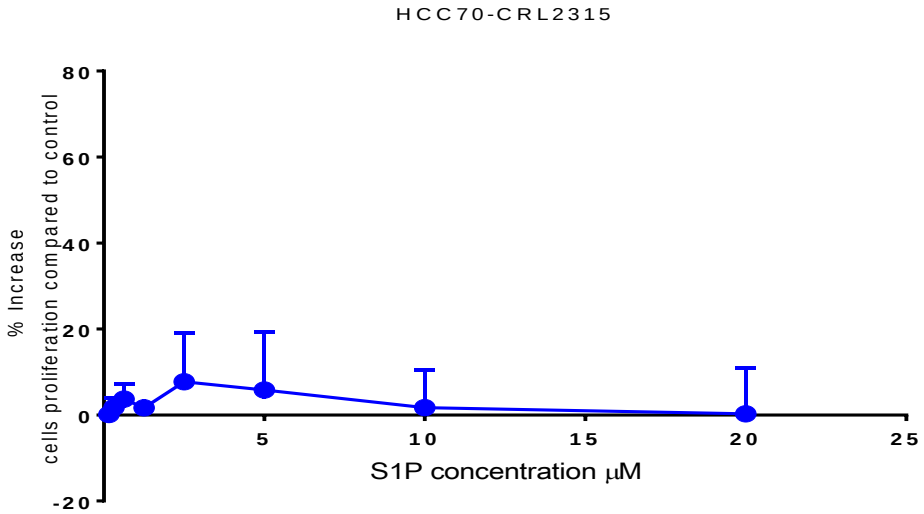


HCC1143-CRL2321




MDA-MB-468HTB-132
% Increase of cells proliferation compared to control
80
60
40
20
0
-20
5
10
15
20
25
S1P concentration μM


HCC38-CRL2314
% Increase of cells proliferation compared to control
80
60
40
20
0
-20
5
10
15
20
25
S1P concentration μM


HCC70-CRL2315
% Increase cells proliferation compared to control
80
60
40
20
0
-20
5
10
15
20
25
S1P concentration μM

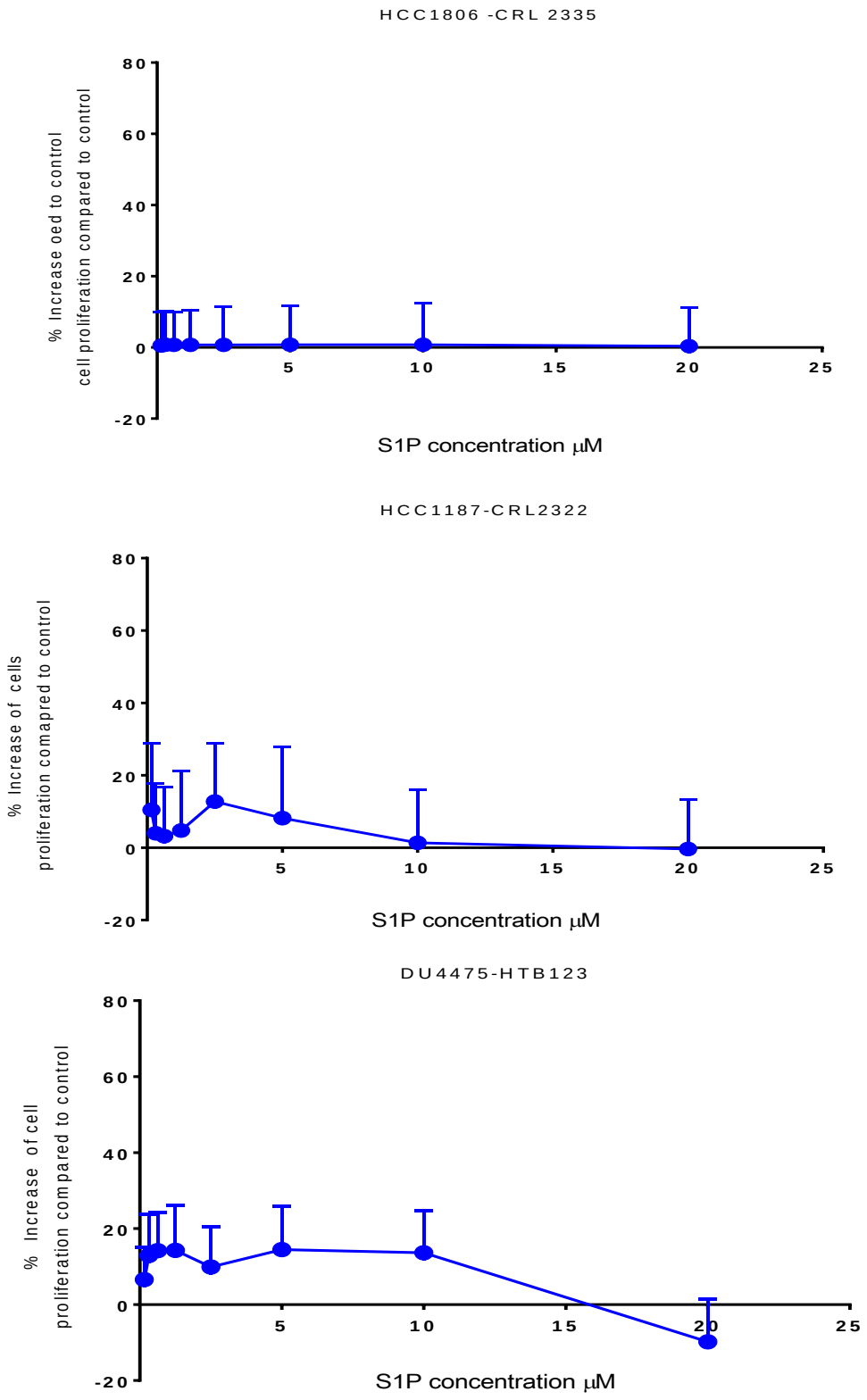


Figure4 : Percent increase of cellular proliferation (mean± SD) compared to control in TNBC/BLBC cell lines after being treated with different concentrations of S1P. Cells treated with the complete growth medium and solvents were used as a control. The mean±SD was calculated from three independent experiments.

4.3 APOPTOSIS STUDIES

Based on the results from the previous sections, I tried to assess the inhibitory effect of S1P and determine whether S1P induces apoptosis using the Annexin V assay. S1P inhibited proliferation in all nine cell lines after 72 hr. Treating cells with S1P caused the cell to detach, shrink and round as shown in the Figure 7.

Cell line HCC1599 showed the highest response to S1P treatment with inhibition of about 55 percent and the results were statistically significant compared to untreated cells (p-value< 0.05). HCC038 showed inhibition of growth ranging from 20- 30 % and the difference between the treated cell and untreated cell was significant (p –value < 0.05). In HCC1806, S1P induced apoptosis at about 20%. However, this inhibition was not significant compared to non-treated cells. As reported in cytotoxicity part, this cell line was least responsive to S1P treatment.

Table 2: Summary of cytotoxicity, proliferation, and apoptosis tests in TNBC/BLBC cell lines

Cell Line	Cytotoxicity	Proliferation	Apoptosis
HCC1599-CRL2331	64% (0.1µM)	17% (2.5µM)	56%
HCC1937-CRL2336	70% (1.25µM)	13% (5 µM)	33%
HCC1143-CRL2321	31% (10µM)	15% (2.5µM)	20%
MDA-MB-468	24% (0.1 µM)	16% (0.3µM)	36%
HCC38-CRL2314	21% (0.3µM)	2% (0.1µM)	33%
HCC70-CRL2315	37% (5µM)	7% (2.5µM)	27%
HCC1806-CRL2335	8%	2%	30%
HCC1187-CRL2322	28% (2.5µM)	12% (1.25µM)	19%
DU4475-HTB123	40% (0.3µM)	14% (5µM)	28%

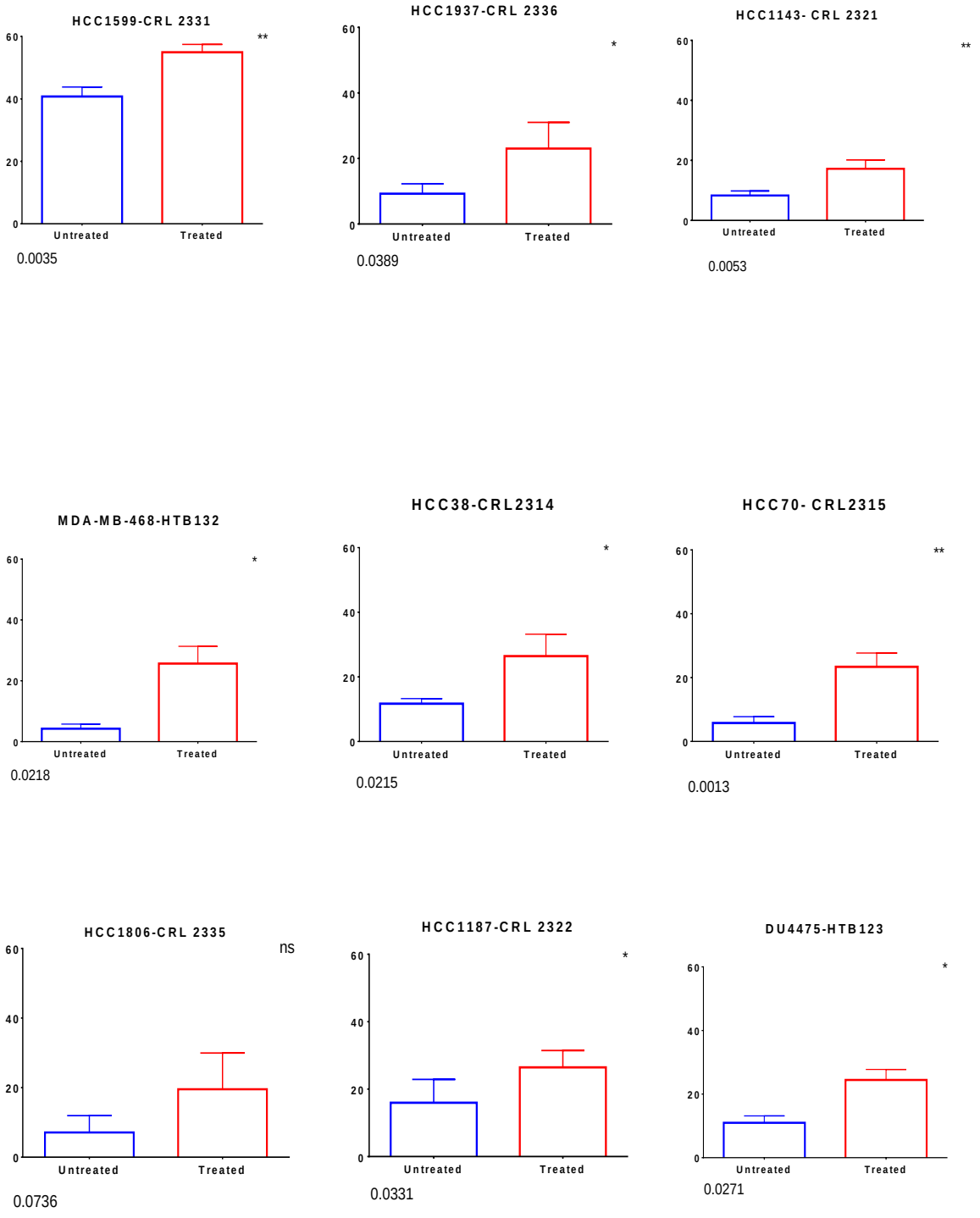
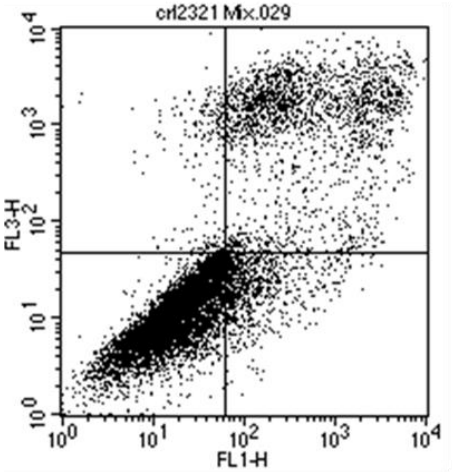
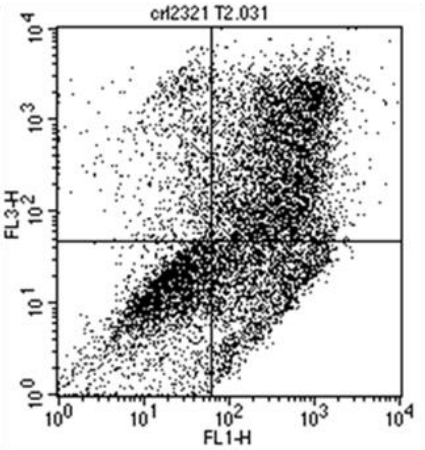


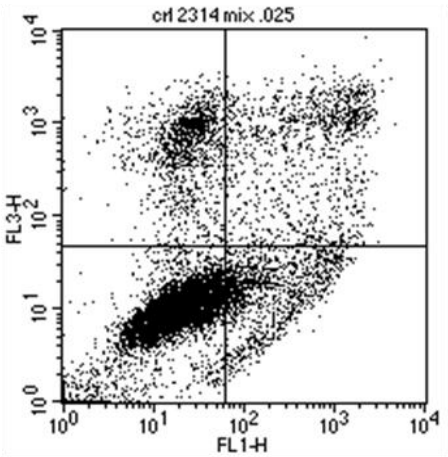
Figure5 : Percent apoptotic cell death using flow cytometry method comprising non-treated cell with S1P treated cell after 72 hr. Cells treated with complete growth medium and solvent was sued as control. Annexin V apoptotic assay was used as described in methods section the percent of cell death was calculated from three independent experiments N = 3, *=P<0.05. ***=P<0.001. p-values are shown under each figure.

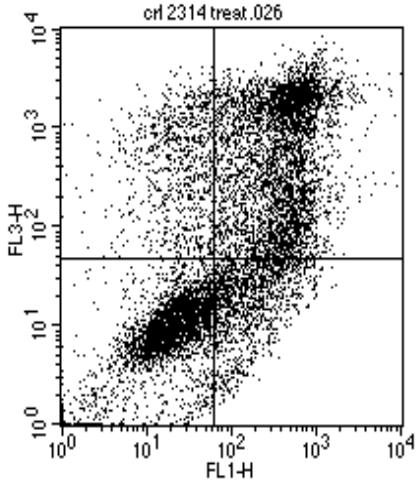
CRL2321



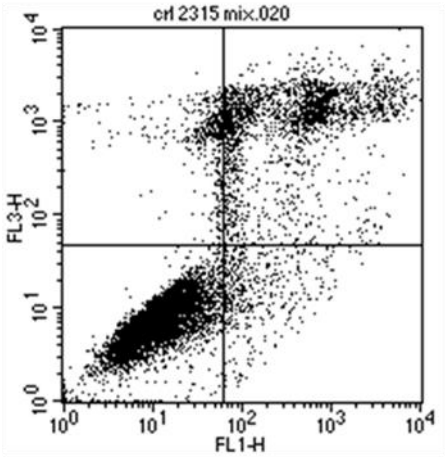


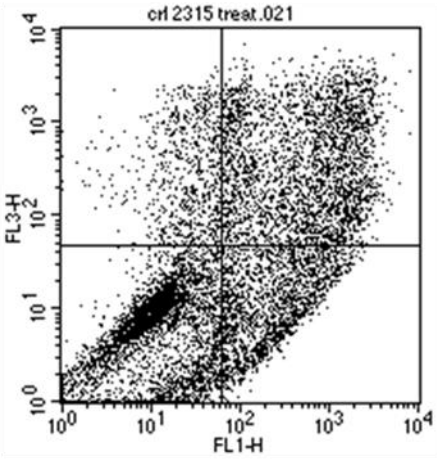
Crl2314



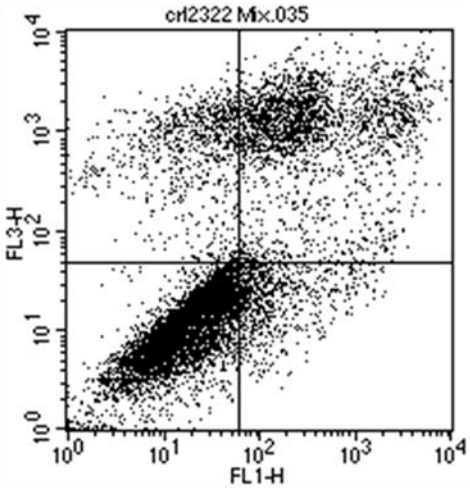


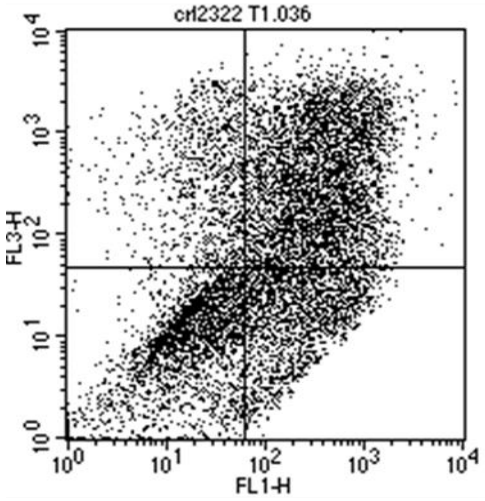
CRL2315



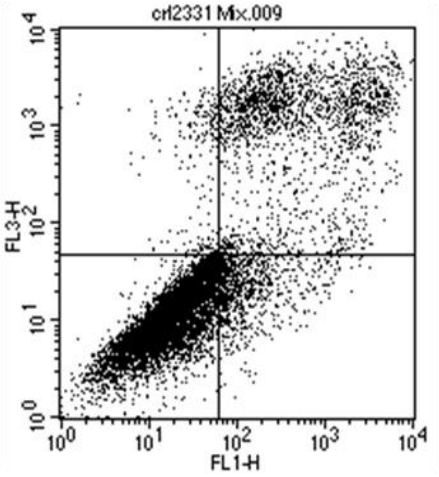


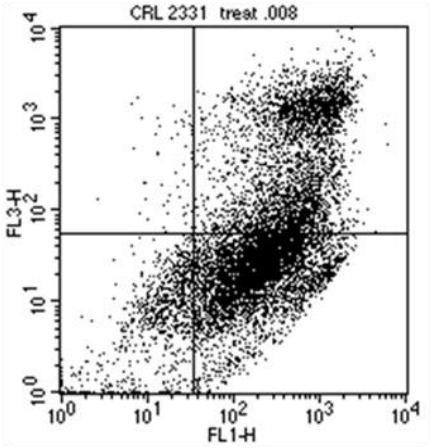
CRL 2322



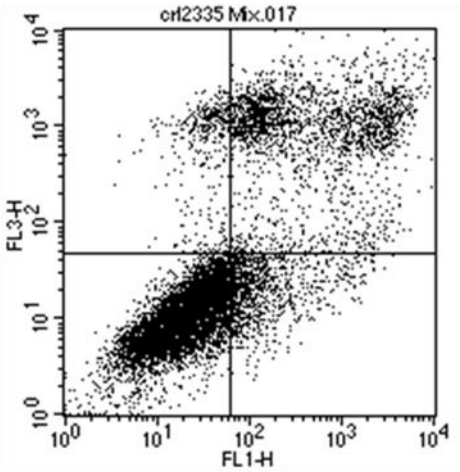


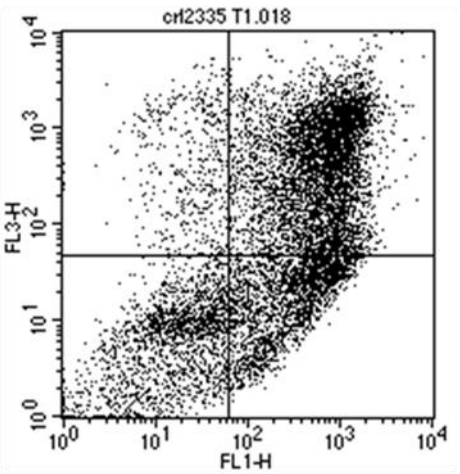
CRL 2331





CRL2335





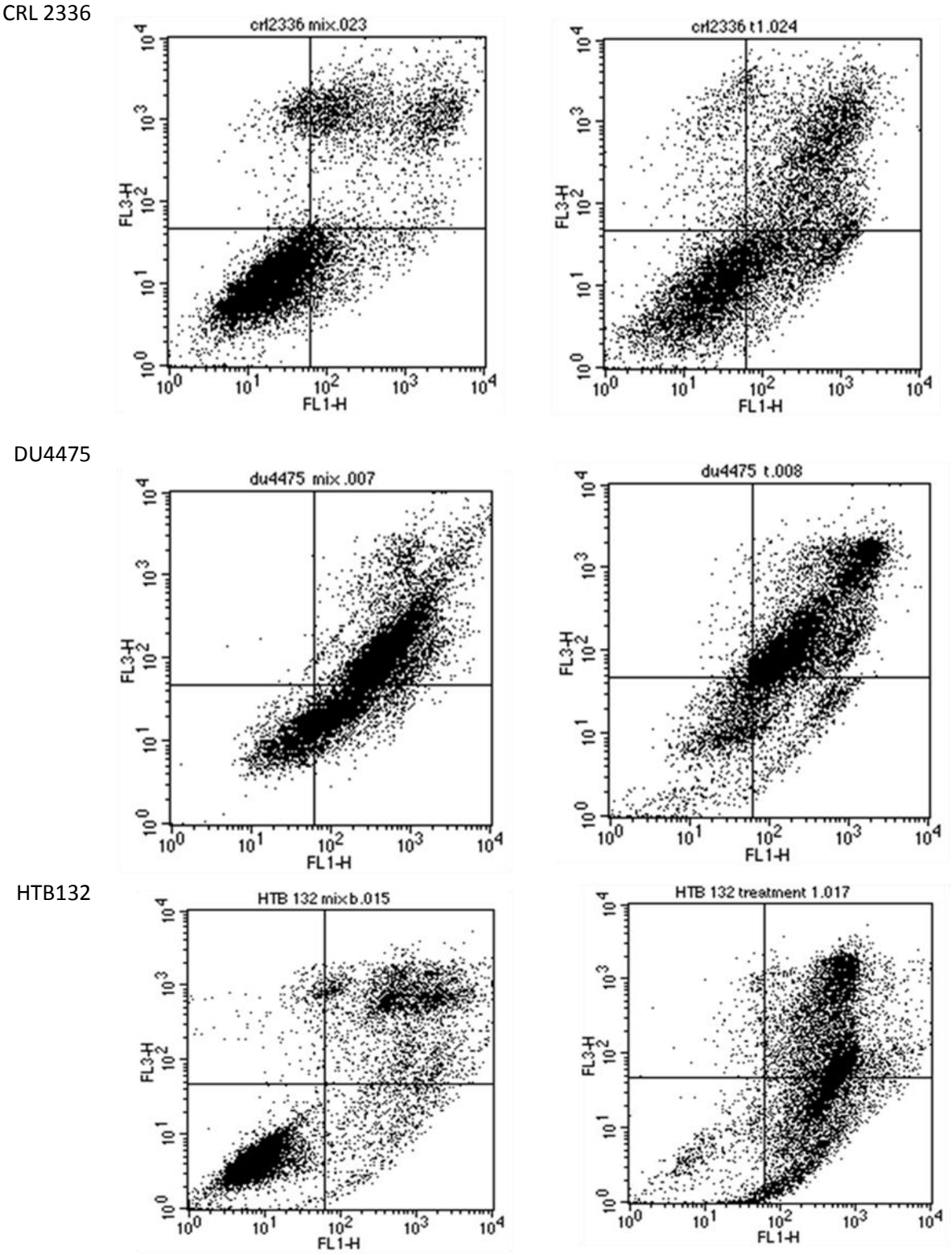
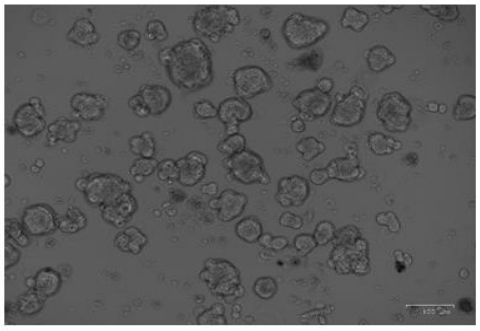


Figure6: Flow cytometry analysis of the nine TNBC/BLBC cell lines after being treated with different concentration of S1P compared to non-treated cells after 72 hrs. Cells treated with complete growth medium and solvent was used as control. Annexin V apoptotic assay was used as described in methods section the percent of cell death was calculated from three independent experiments N = 3

HCC1599-CRL 2331

Non Treated



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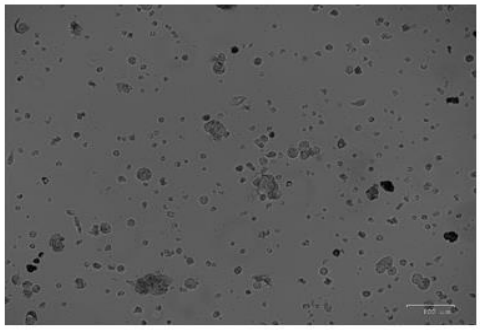

HCC1937-CRL 2336

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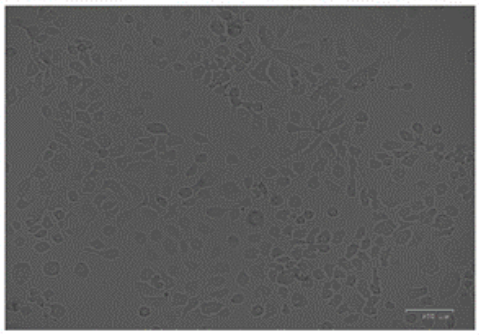

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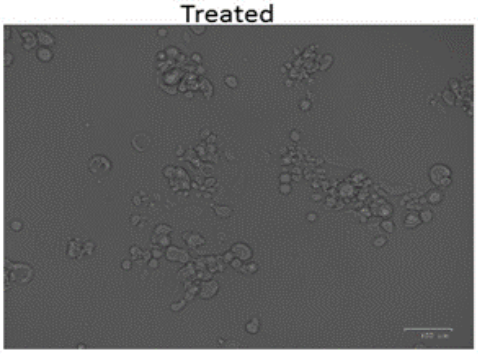

HCC1143- CRL 2321

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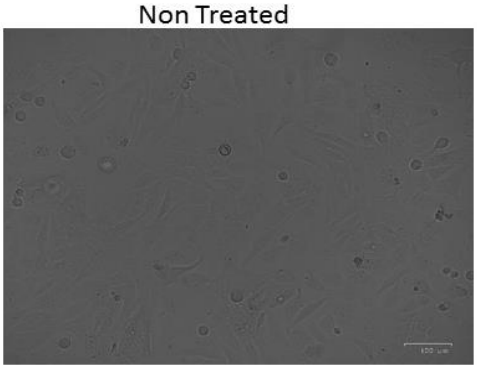

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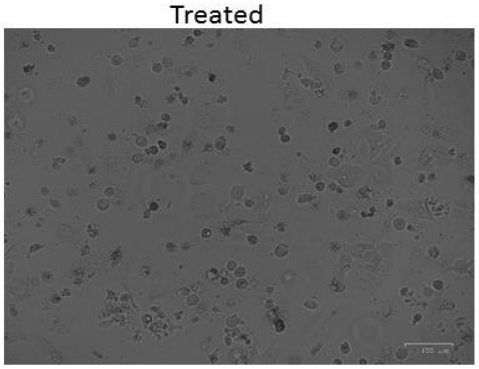
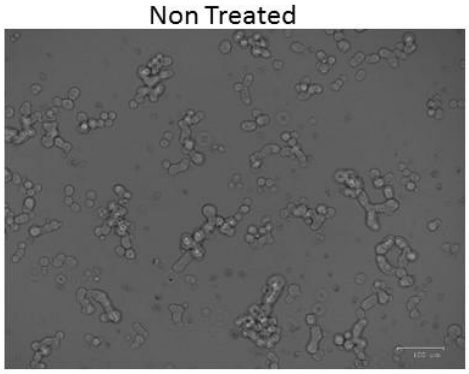
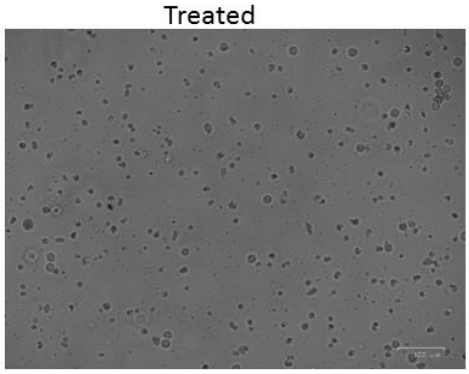


DU 4475- HTB123

Non Treated

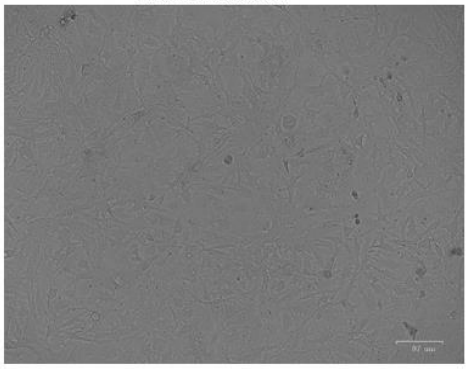


Treated



HCC38-CRL 2314

Non Treated



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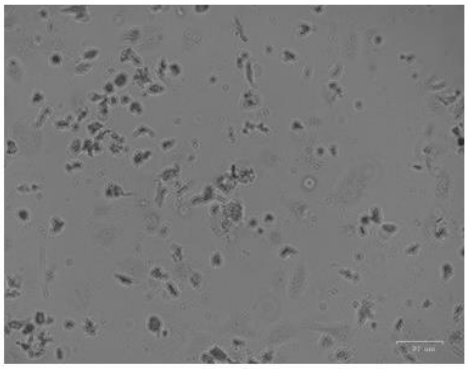
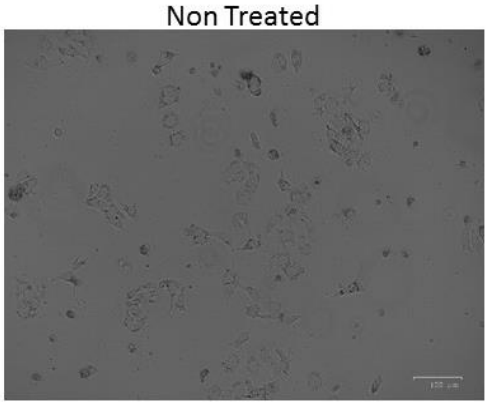
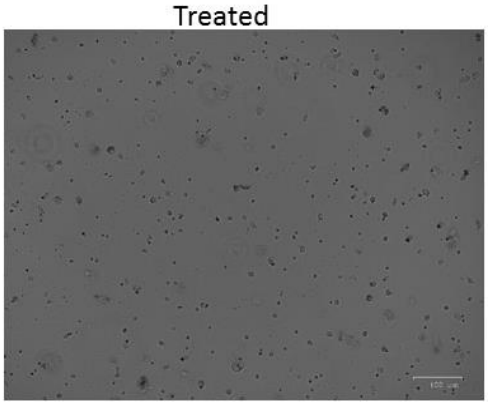


HCC1187-CRL 2322

Non Treated



Treated



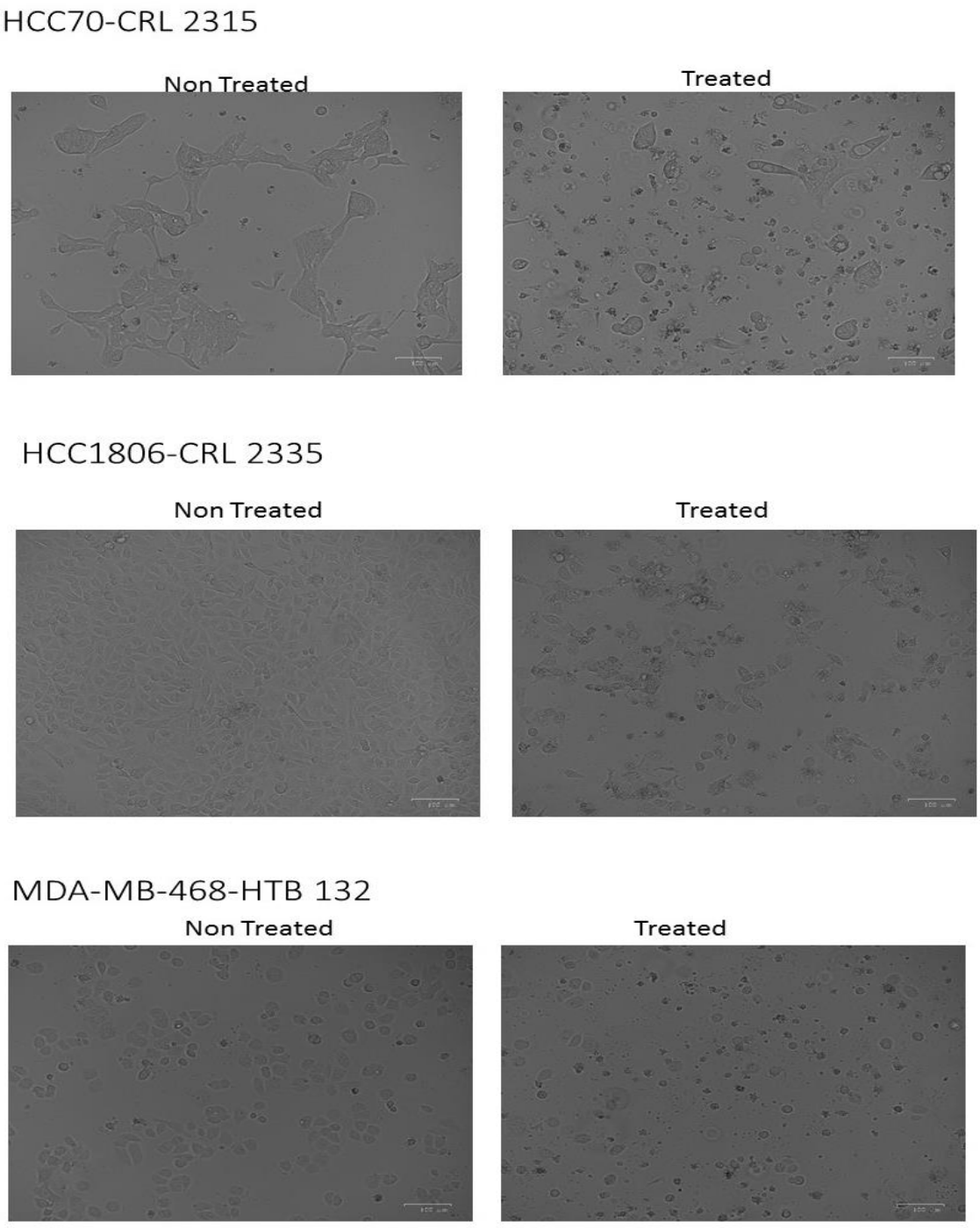


Figure 7: Nine TNBC/BLBC cell lines after 72 hr treatment with different S1P concentration compared to untreated cells. S1P caused the cell to shrink, round and detached. ZOETM Fluorescent cell image from Bio-Rad was used to take these images. Cells treated with complete growth medium and solvent was used as control.

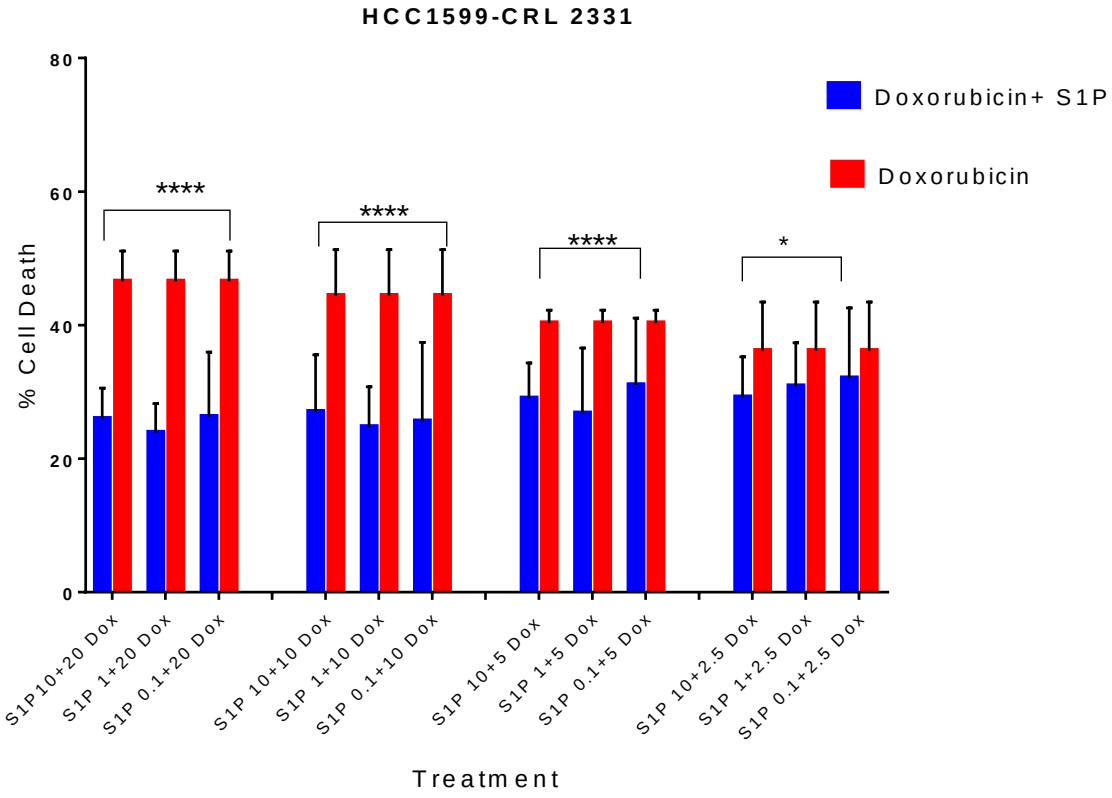
4.4 CHEMOTHERAPY COMBINATION STUDIES

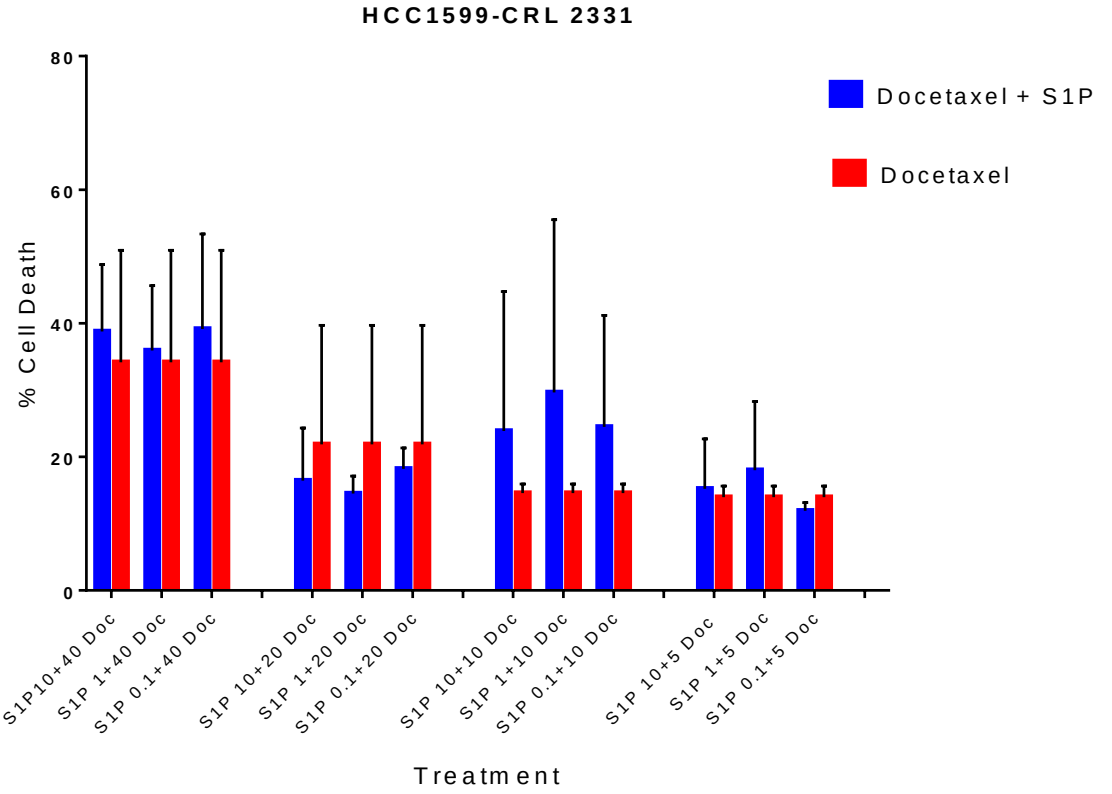
The combinations of the chemotherapeutic agent docetaxel (DOC) and doxorubicin (DOX) with S1P were evaluated for 72 hr in the nine cell lines.

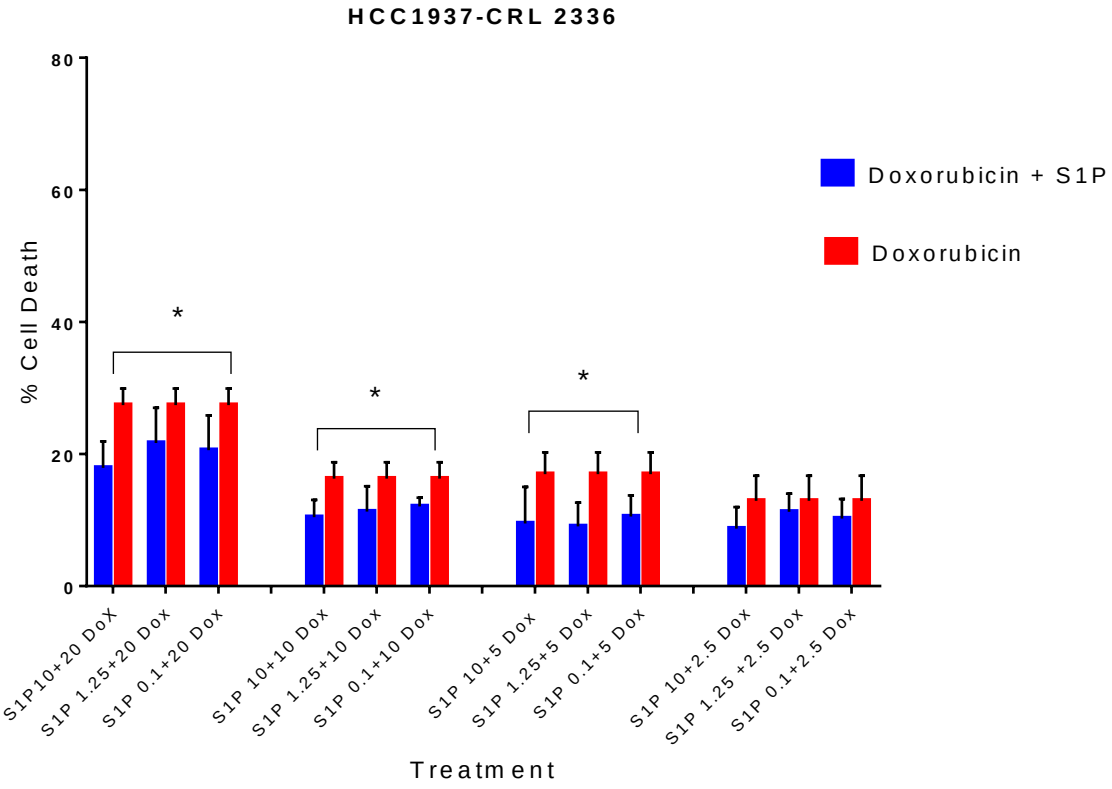
Each cell line responds differently to treatments. Combination studies of DOX with S1P in HCC1599, showed a reduction in cell death compared to DOX treatment alone as shown in Figure 8 (p-value < 0.0001). Maximum cell death achieved by DOX alone was around 45% at a concentration of 20 µM while when combined with S1P the cell death dropped to around 25 %. The impact of S1P co-treatment with DOC in the same cell line did not show any difference from DOC treatment alone. Although the combination showed higher cell death at 40µM and 10µM DOC, the difference was not statistically significant (p-value > 0.05). In HCC1937, S1P co-treatment with DOX resulted in a significant decrease in the percentage of cell death at concentrations, 20, 10 and 5µM (P-value<0.05) but not at 2.5µM. S1P co-treatment with DOC showed no significant difference in the percentage of cell death at any concentration. The DOC treatment showed higher cell death compared to DOX treatment. HCC1806 cell line showed no significant difference between the chemotherapy alone as compared with the combination for both DOC and DOX. However, DOX cytotoxicity results show that this cell line is more responsive to DOX than DOC (P-value < 0.05) is. HCC1187 cell line results show a significant decrease in the percentage of cell death for both DOX and DOC when co-administered with S1P. DOX combination showed significant reduction in the cytotoxic effect at concentration of 20, 10, 5µM (p value< 0.05) and no significant difference at 2.5µM. DOC combination showed significant reduction at 40, 20, 10µM but no significant reduction at 5µM. The results also show that this cell line is more responsive to DOC than DOX (p value<0.05). HCC1143 combination of DOX with S1P results showed no significant advantage of the combination over the chemotherapy

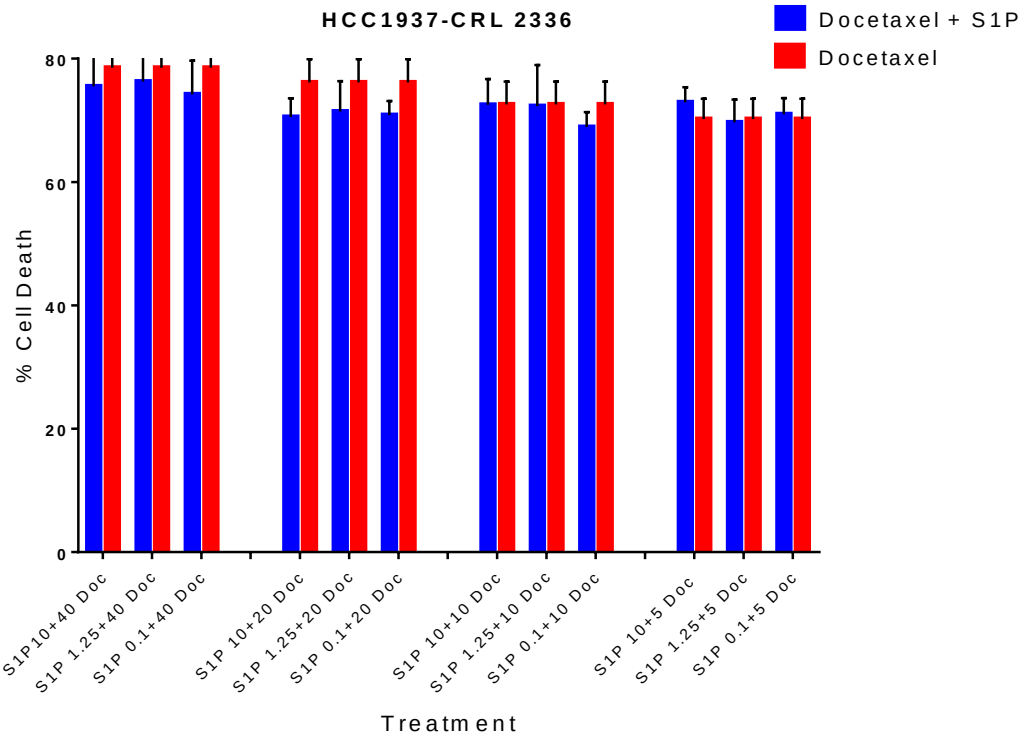
treatment alone for all concentrations (p-value<0.05). DOC combination showed more reduction in the percentage of cell death at high DOC concentration (40 and 20 µM, p-value<0.05). However, at low DOC concentrations there was no significant difference. No significant difference was observed between the DOC and DOX treatments.

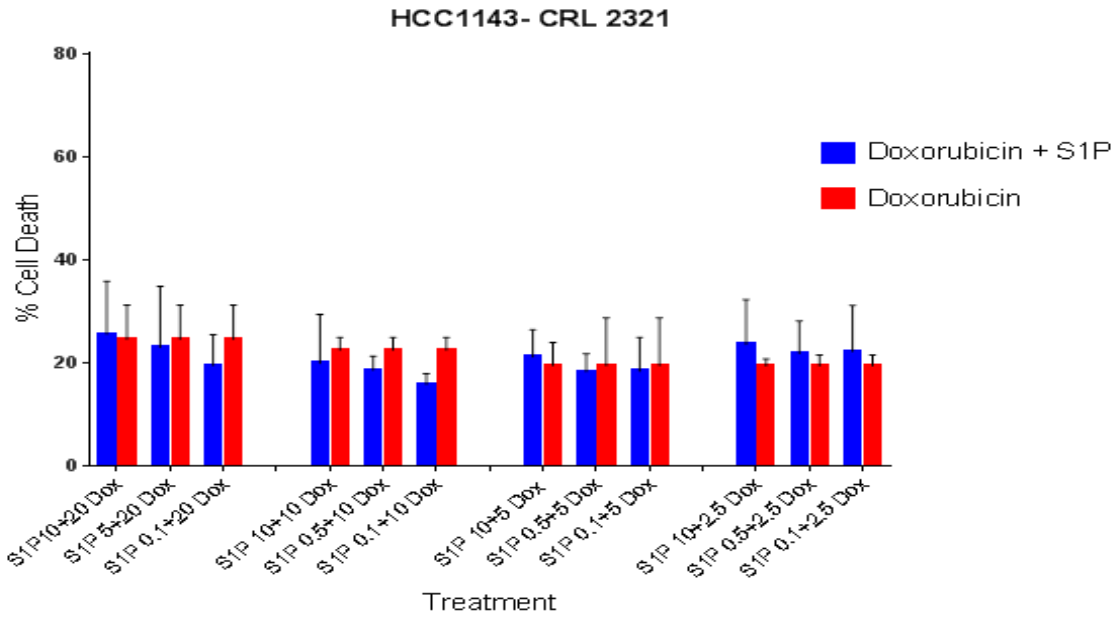
MDA-MB-468 co-administration of S1P with DOX resulted in a more significant reduction in the percentage of cell death and the difference was more pronounced at higher concentration (p-value<0.0001). The combination of S1P with DOC showed no significant result over DOC treatment alone. MDA-MB-468 cell line shows a more significant response to DOX over DOC (p-value <0.05). HCC38, ATCC CRL-2314 S1P combination with DOX showed an increase in the number of dead cells at high concentrations. However, this increase was not significant (p-value> 0.05) and at a lower concentration the combination showed a reduction in the number of dead cells (P value< 0.05). DOC combination showed no advantage in increasing percentage of cell death. The difference in response to DOX treatment and DOC was not significant in this cell line. The combination of S1P and DOX in HCC70 did not show an increase in number of dead cells in this cell line. The combination of S1P with DOC showed a significant reduction in the number of the dead cells at higher concentration (P-value <0.05). DOC treatment produced a cell death of about 77 % while the DOX treatment achieved a maximum of 22 %. For DU4475, S1P-DOX combination showed significant reduction in the number of dead cells at higher concertation while at lower concentration the number of dead cells was not significantly different from DOX treatment alone. DOC combination with S1P did not show any significant difference from DOC treatment alone. Both DOX and DOC produced approximately the same percentage of dead cells in DU4475 cell line.

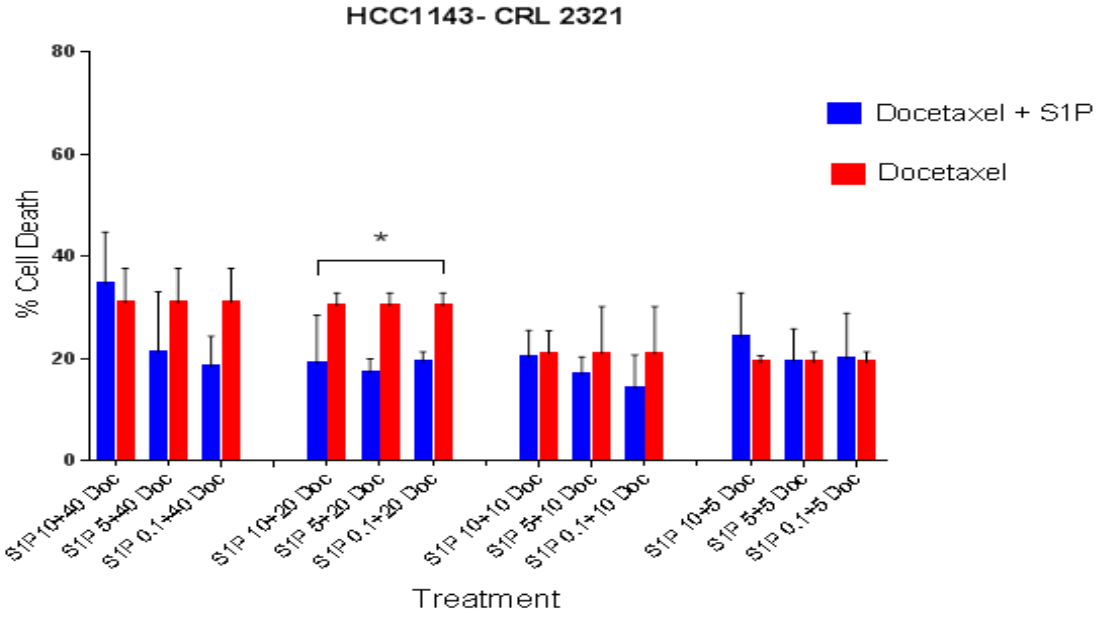

HCC1599-CRL 2331
Doxorubicin+ S1P
Doxorubicin
% Cell Death
80
60
40
20
0
****
****
****
*
S1P10+20 Dox
S1P 1+20 Dox
S1P 0.1+20 Dox
S1P 10+10 Dox
S1P 1+10 Dox
S1P 0.1+10 Dox
S1P 10+5 Dox
S1P 1+5 Dox
S1P 0.1+5 Dox
S1P 10+2.5 Dox
S1P 1+2.5 Dox
S1P 0.1+2.5 Dox
Treatment

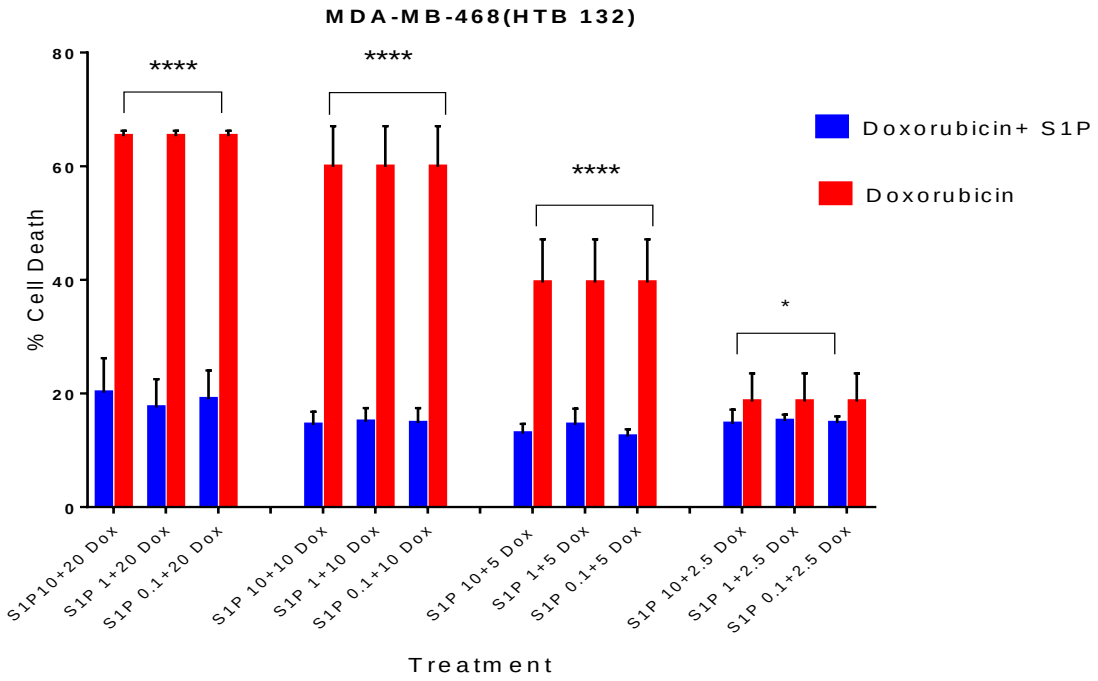

HCC1599-CRL 2331
Docetaxel + S1P
Docetaxel
% Cell Death
80
60
40
20
0
S1P10+40 Doc
S1P 1+40 Doc
S1P 0.1+40 Doc
S1P 10+20 Doc
S1P 1+20 Doc
S1P 0.1+20 Doc
S1P 10+10 Doc
S1P 1+10 Doc
S1P 0.1+10 Doc
S1P 10+5 Doc
S1P 1+5 Doc
S1P 0.1+5 Doc
Treatment

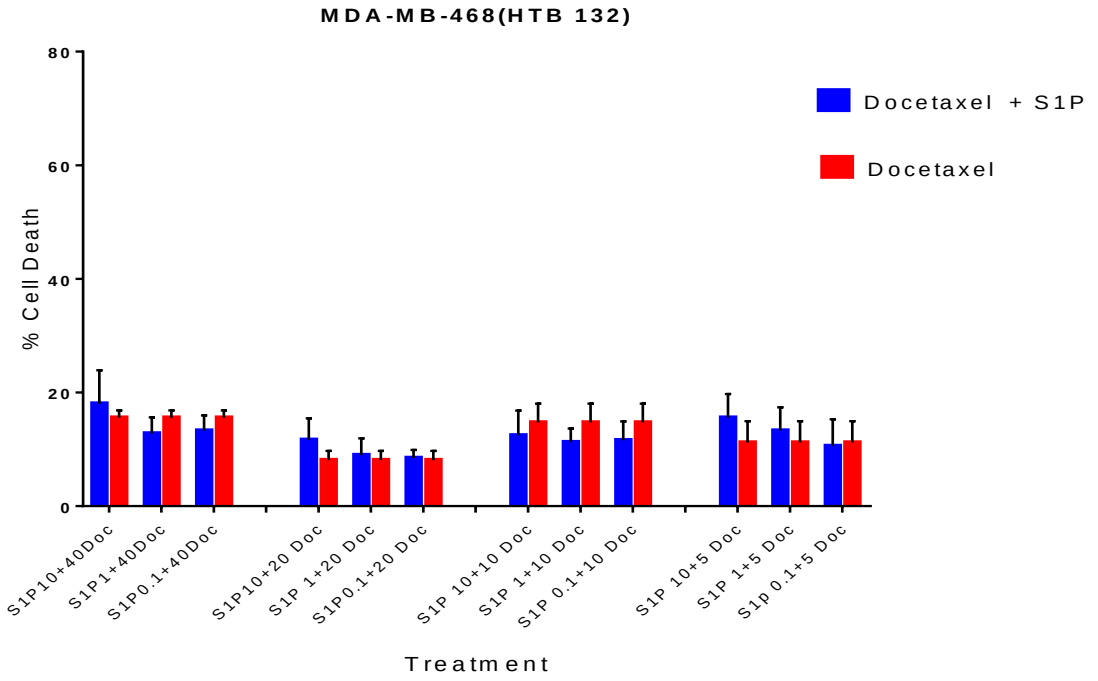

HCC1937-CRL 2336
Doxorubicin + S1P
Doxorubicin
% Cell Death
80
60
40
20
0
*
*
*
S1P10+20 DoX
S1P 1.25+20 Dox
S1P 0.1+20 Dox
S1P 10+10 Dox
S1P 1.25+10 Dox
S1P 0.1+10 Dox
S1P 10+5 Dox
S1P 1.25+5 Dox
S1P 0.1+5 Dox
S1P 10+2.5 Dox
S1P 1.25 +2.5 Dox
S1P 0.1+2.5 Dox
Treatment

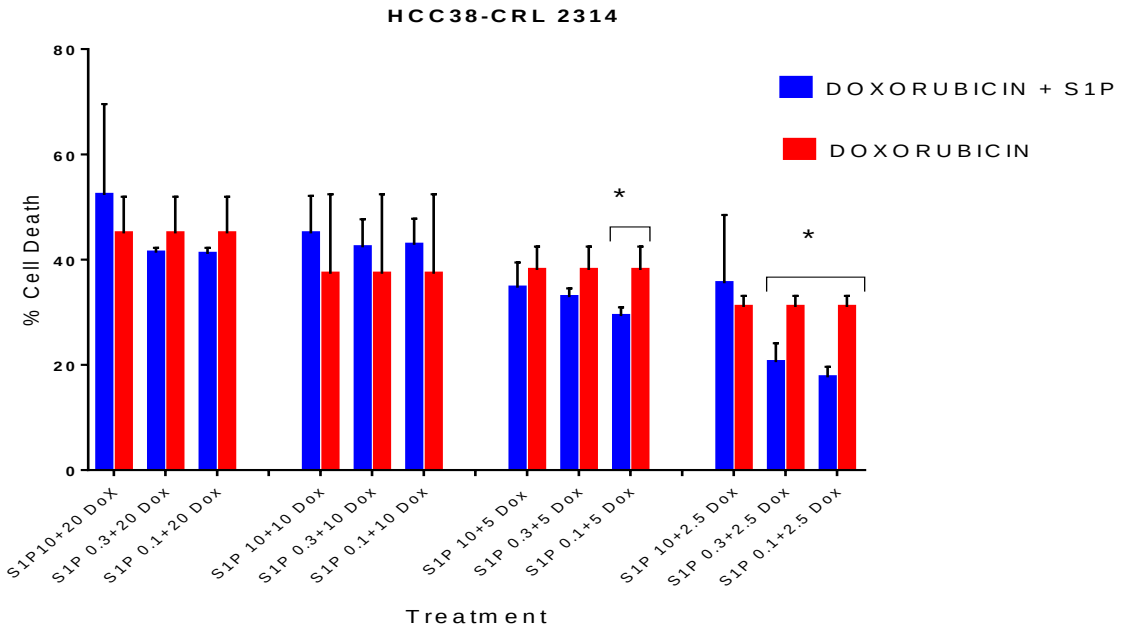

HCC1937-CRL 2336
Docetaxel + S1P
Docetaxel
% Cell Death
80
60
40
20
0
S1P10+40 Doc
S1P 1.25+40 Doc
S1P 0.1+40 Doc
S1P 10+20 Doc
S1P 1.25+20 Doc
S1P 0.1+20 Doc
S1P 10+10 Doc
S1P 1.25+10 Doc
S1P 0.1+10 Doc
S1P 10+5 Doc
S1P 1.25+5 Doc
S1P 0.1+5 Doc
Treatment

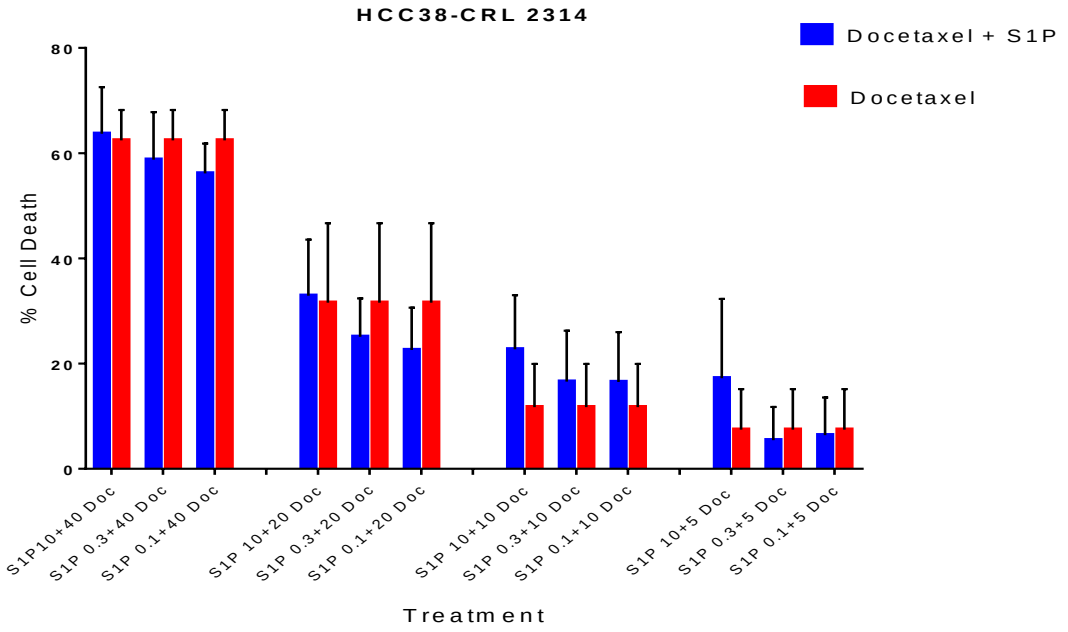

HCC1143- CRL 2321
% Cell Death
80
60
40
20
0
Doxorubicin + S1P
Doxorubicin
S1P 10+20 Dox
S1P 5+20 Dox
S1P 0.1+20 Dox
S1P 10+10 Dox
S1P 0.5+10 Dox
S1P 0.1+10 Dox
S1P 10+5 Dox
S1P 0.5+5 Dox
S1P 0.1+5 Dox
S1P 10+2.5 Dox
S1P 0.5+2.5 Dox
S1P 0.1+2.5 Dox
Treatment

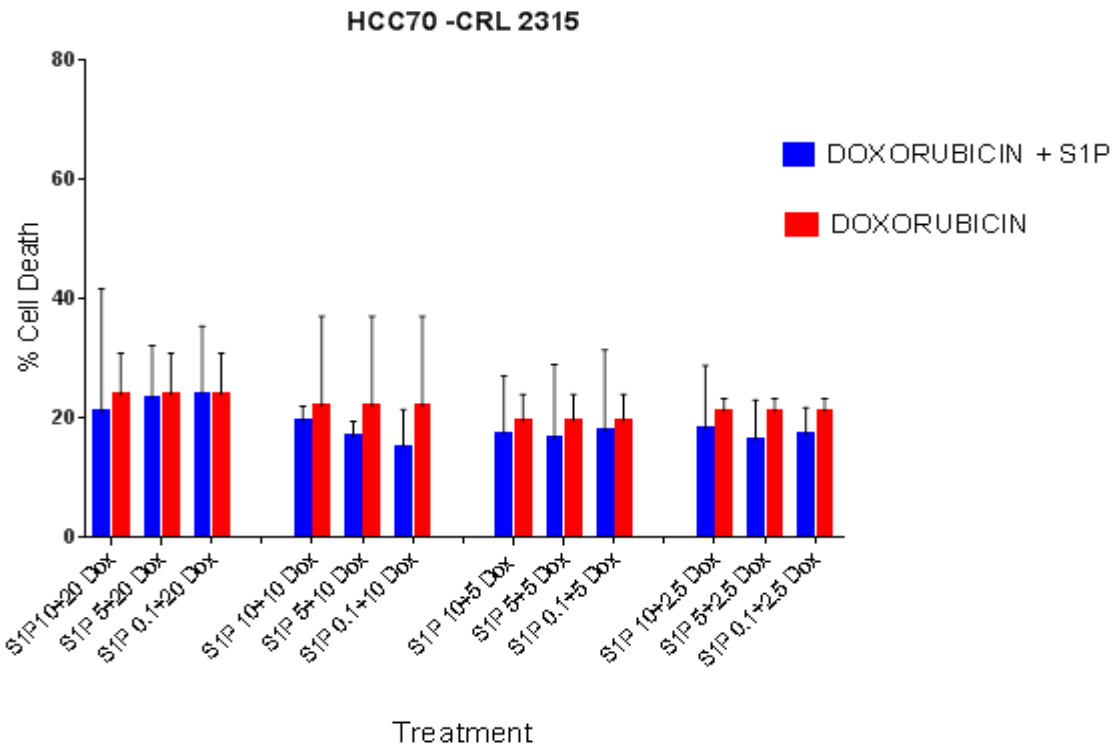

HCC1143- CRL 2321
% Cell Death
80
60
40
20
0
*
Docetaxel + S1P
Docetaxel
S1P 10+40 Doc
S1P 5+40 Doc
S1P 0.1+40 Doc
S1P 10+20 Doc
S1P 5+20 Doc
S1P 0.1+20 Doc
S1P 10+10 Doc
S1P 5+10 Doc
S1P 0.1+10 Doc
S1P 10+5 Doc
S1P 5+5 Doc
S1P 0.1+5 Doc
Treatment

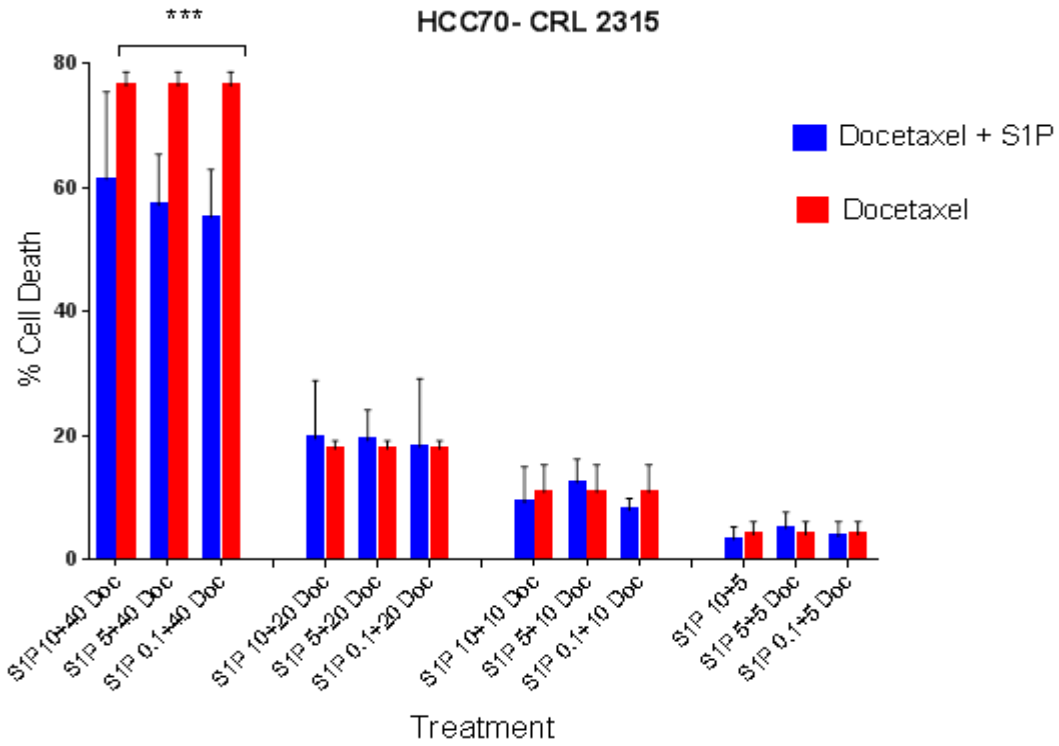

MDA-MB-468(HTB 132)
% Cell Death
80
60
40
20
0
****
****
****
*
Doxorubicin+ S1P
Doxorubicin
S1P10+20 Dox
S1P 1+20 Dox
S1P 0.1+20 Dox
S1P 10+10 Dox
S1P 1+10 Dox
S1P 0.1+10 Dox
S1P 10+5 Dox
S1P 1+5 Dox
S1P 0.1+5 Dox
S1P 10+2.5 Dox
S1P 1+2.5 Dox
S1P 0.1+2.5 Dox
Treatment

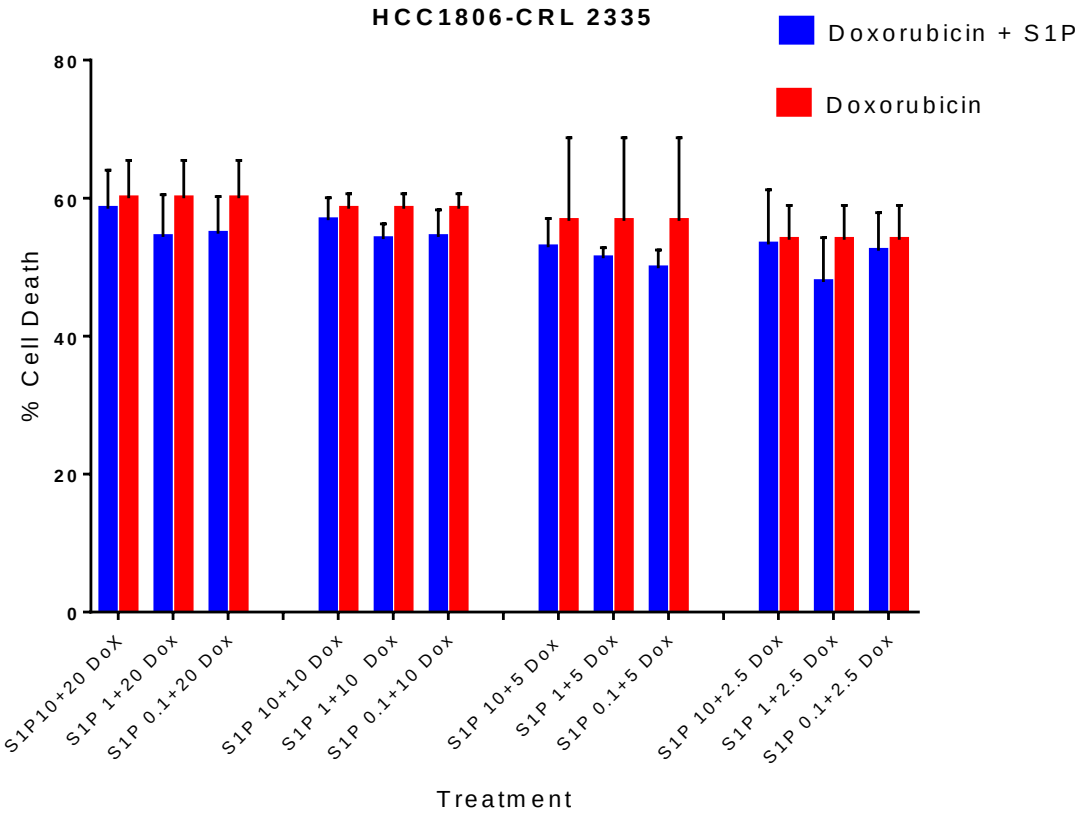

MDA-MB-468(HTB 132)
% Cell Death
80
60
40
20
0
Docetaxel + S1P
Docetaxel
S1P10+40Doc
S1P1+40Doc
S1P0.1+40Doc
S1P10+20 Doc
S1P 1+20 Doc
S1P0.1+20 Doc
S1P 10+10 Doc
S1P 1+10 Doc
S1P 0.1+10 Doc
S1P 10+5 Doc
S1P 1+5 Doc
S1p 0.1+5 Doc
Treatment

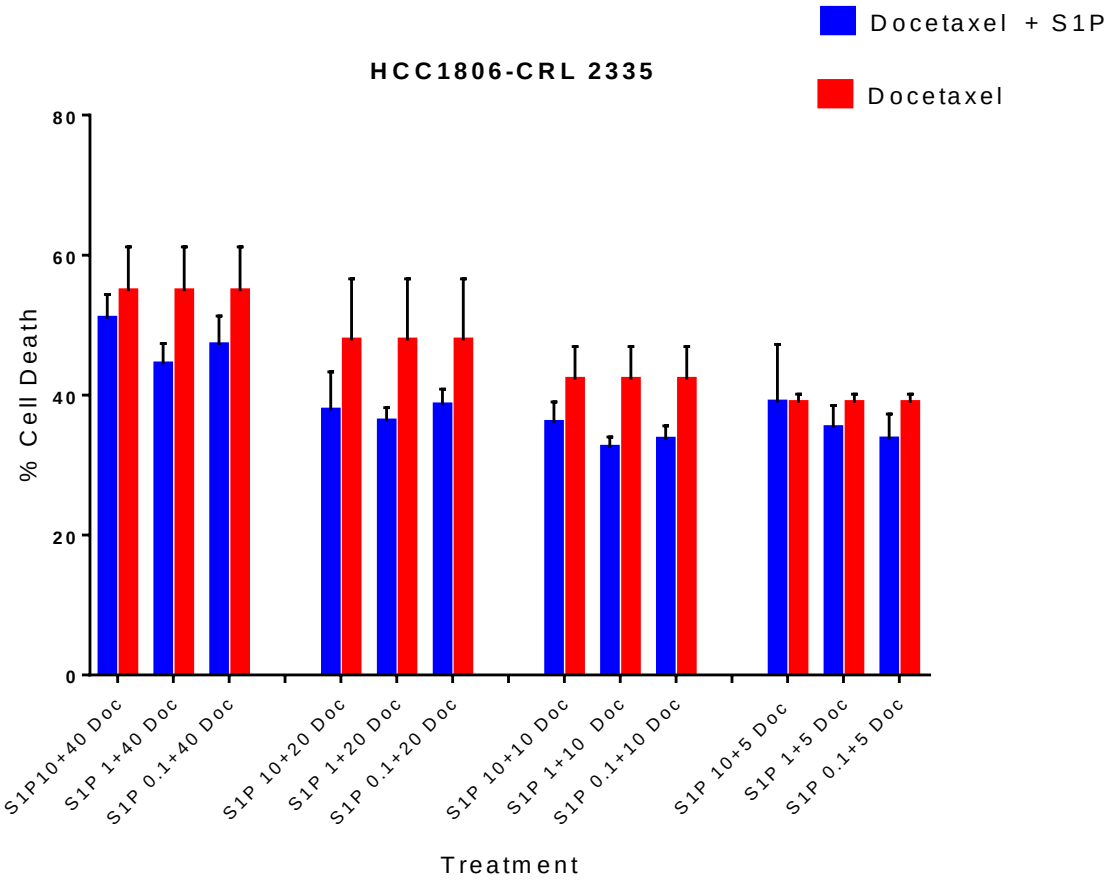

HCC38-CRL 2314
DOXORUBICIN + S1P
DOXORUBICIN
% Cell Death
80
60
40
20
0
*
*
S1P10+20 DoX
S1P 0.3+20 Dox
S1P 0.1+20 Dox
S1P 10+10 Dox
S1P 0.3+10 Dox
S1P 0.1+10 Dox
S1P 10+5 Dox
S1P 0.3+5 Dox
S1P 0.1+5 Dox
S1P 10+2.5 Dox
S1P 0.3+2.5 Dox
S1P 0.1+2.5 Dox
Treatment

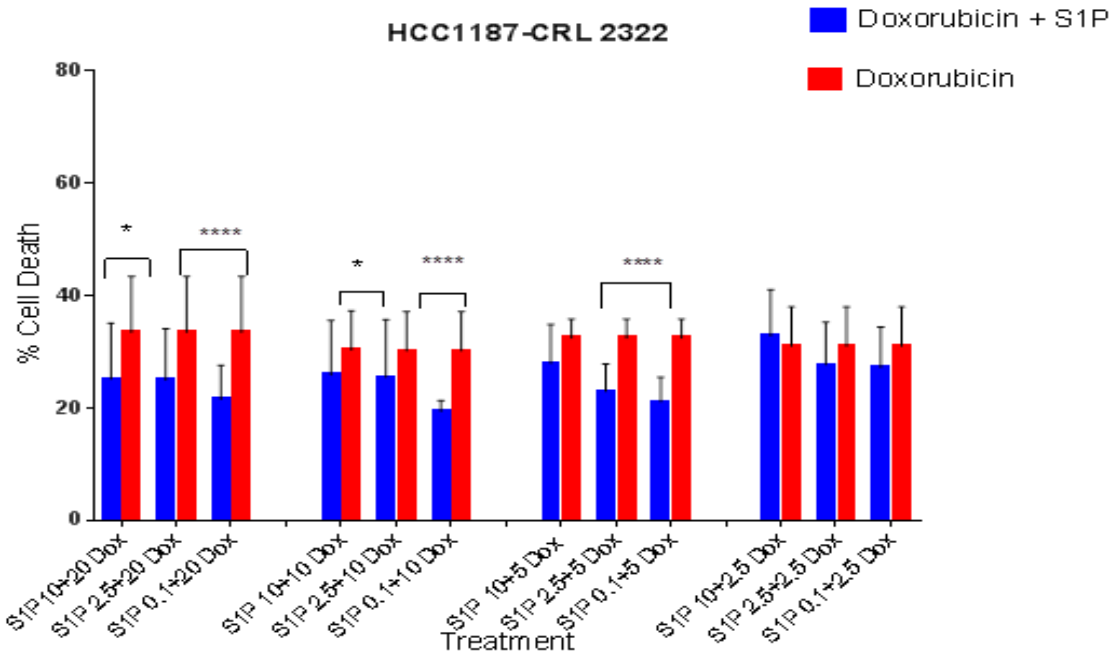

HCC38-CRL 2314
Docetaxel + S1P
Docetaxel
% Cell Death
80
60
40
20
0
S1P10+40 Doc
S1P 0.3+40 Doc
S1P 0.1+40 Doc
S1P 10+20 Doc
S1P 0.3+20 Doc
S1P 0.1+20 Doc
S1P 10+10 Doc
S1P 0.3+10 Doc
S1P 0.1+10 Doc
S1P 10+5 Doc
S1P 0.3+5 Doc
S1P 0.1+5 Doc
Treatment

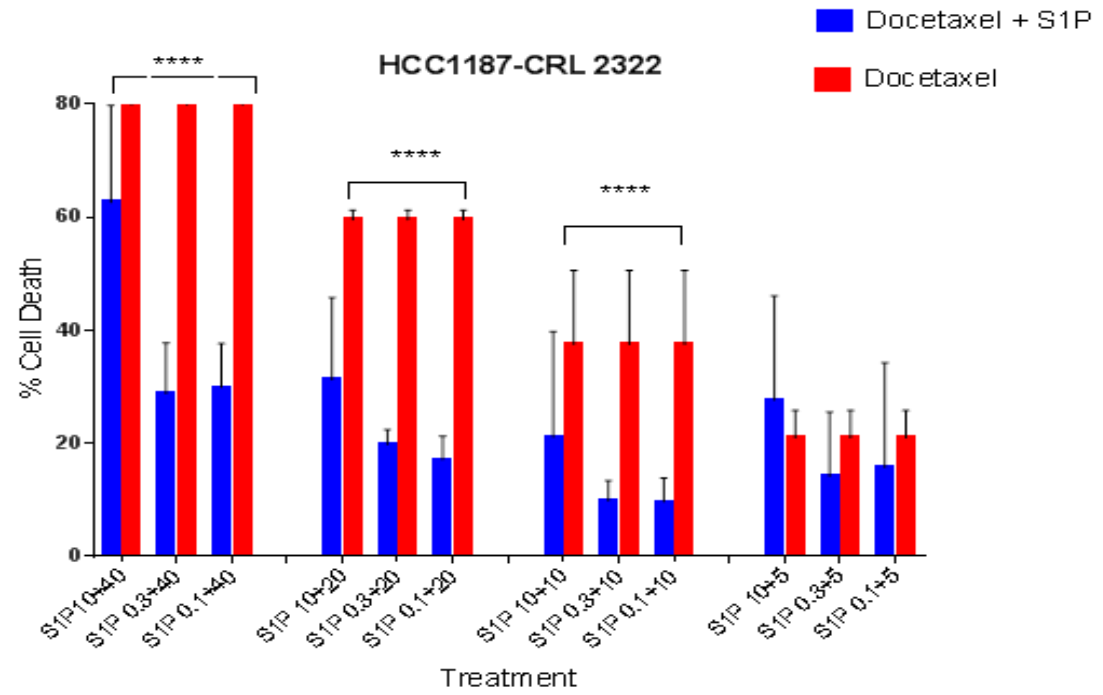

HCC70 -CRL 2315
% Cell Death
80
60
40
20
0
DOXORUBICIN + S1P
DOXORUBICIN
S1P 10+20 Dox
S1P 5+20 Dox
S1P 0.1+20 Dox
S1P 10+10 Dox
S1P 5+10 Dox
S1P 0.1+10 Dox
S1P 10+5 Dox
S1P 5+5 Dox
S1P 0.1+5 Dox
S1P 10+2.5 Dox
S1P 5+2.5 Dox
S1P 0.1+2.5 Dox
Treatment


***
HCC70- CRL 2315
% Cell Death
80
60
40
20
0
Docetaxel + S1P
Docetaxel
S1P 10+40 Doc
S1P 5+40 Doc
S1P 0.1+40 Doc
S1P 10+20 Doc
S1P 5+20 Doc
S1P 0.1+20 Doc
S1P 10+10 Doc
S1P 5+10 Doc
S1P 0.1+10 Doc
S1P 10+5
S1P 5+5 Doc
S1P 0.1+5 Doc
Treatment


HCC1806-CRL 2335
Doxorubicin + S1P
Doxorubicin
% Cell Death
0
20
40
60
80
S1P10+20 DoX
S1P 1+20 Dox
S1P 0.1+20 Dox
S1P 10+10 Dox
S1P 1+10 Dox
S1P 0.1+10 Dox
S1P 10+5 Dox
S1P 1+5 Dox
S1P 0.1+5 Dox
S1P 10+2.5 Dox
S1P 1+2.5 Dox
S1P 0.1+2.5 Dox
Treatment


Docetaxel + S1P
Docetaxel
HCC1806-CRL 2335
% Cell Death
0
20
40
60
80
S1P10+40 Doc
S1P 1+40 Doc
S1P 0.1+40 Doc
S1P 10+20 Doc
S1P 1+20 Doc
S1P 0.1+20 Doc
S1P 10+10 Doc
S1P 1+10 Doc
S1P 0.1+10 Doc
S1P 10+5 Doc
S1P 1+5 Doc
S1P 0.1+5 Doc
Treatment


HCC1187-CRL 2322
Doxorubicin + S1P
Doxorubicin
% Cell Death
Treatment
S1P 10+20 Dox
S1P 2.5+20 Dox
S1P 0.1+20 Dox
S1P 10+10 Dox
S1P 2.5+10 Dox
S1P 0.1+10 Dox
S1P 10+5 Dox
S1P 2.5+5 Dox
S1P 0.1+5 Dox
S1P 10+2.5 Dox
S1P 2.5+2.5 Dox
S1P 0.1+2.5 Dox


Docetaxel + S1P
Docetaxel
HCC1187-CRL 2322
% Cell Death
Treatment
S1P10+40
S1P 0.3+40
S1P 0.1+40
S1P 10+20
S1P 0.3+20
S1P 0.1+20
S1P 10+10
S1P 0.3+10
S1P 0.1+10
S1P 10+5
S1P 0.3+5
S1P 0.1+5

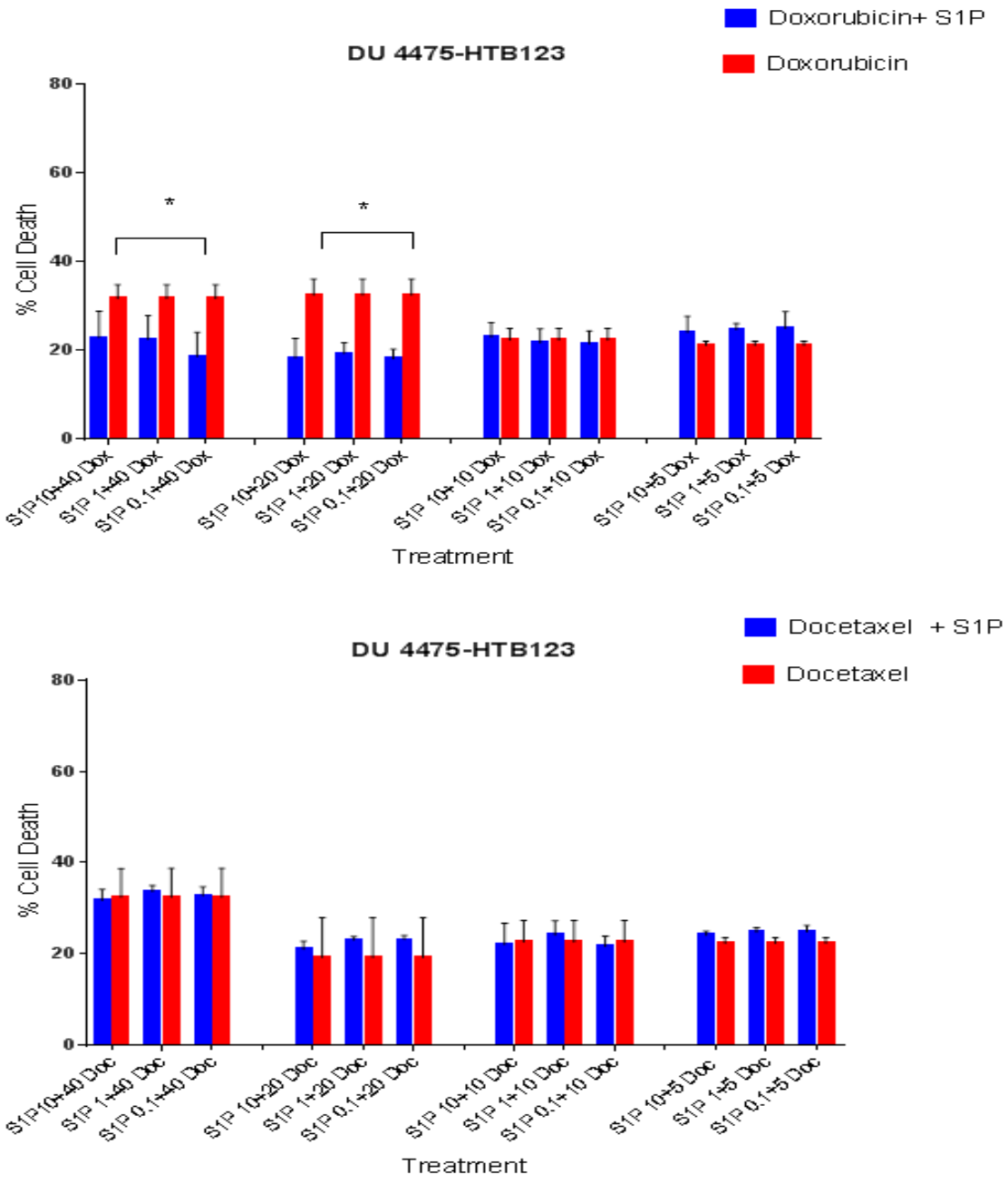


Figure 8: S1P combination with DOX /DOC compared to DOX/DOC alone after 72 hr. incubation in TNBC/BLBC cell lines. Four different concentrations of the chemotherapeutic drug used with three different concentrations of S1P (10,0.1µM and concentration that produced maximum death in cytotoxicity studies). CytoTox 96® Non-Radioactive Cytotoxicity assay was performed as described in the method section. Cells treated with the complete growth media and the solvent were used as the control. Results are shown as mean ± SD. The mean ± SD was calculated from three independent experiments N = 3, *=P<0.05. ***=P<0.001

5. DISCUSSION

Developing novel agents and tailoring regimens to an individual patient is the main goal of cancer treatment. A selection of appropriate treatments for breast cancer patients requires a better understanding of different subtypes of tumors. TNBCs have poorer prognosis than other subtypes of breast cancer, and risk of recurrence is high. Lack of selectivity of clinically available chemotherapeutic drugs comprises one of the major challenges in cancer treatment. Therefore, research is now being focused to find more selective treatment with fewer side effects on normal cells.

Various intracellular and extracellular biological processes are controlled by S1P. Targeting sphingolipid pathways is being used as therapeutic strategies for the treatment of cancer. S1P is normally reported to increase cell proliferation, migration and angiogenesis in cancer. However, several studies reported the involvement S1P in cell death[92 173].

5.1 SPHINGOSINE-1-PHOSPHATE DUAL EFFECT IN TRIPLE NEGATIVE /BASAL-LIKE BREAST CANCER

The focus of this study is to examine the cytotoxicity of the bioactive lipid, S1P, in TNBC/BLBC cell lines. All nine cell lines are characterized by lacking expression of ER, PR and HER2/neu receptors, and they share basal-like morphology. The present study was undertaken to determine how cancer cells that lack the expression of ER, PR, HER/neu would respond to S1P treatment.

Evaluation of S1P toxicity against TNBC/BLBC cells was done *in-vitro*. For the TNBC/BLBC cell lines, cells do not die immediately after treatment with S1P. For most of the cell lines, significant death occurs after 48 to 72h (Figure 3). Our data demonstrated

greater sensitivity to S1P after 72h treatment for the TNBC/BLBC cell lines. S1P gave a consistent response in all cell lines except HCC1806 cell line, which was less responsive to S1P treatment. This study is the first to show that S1P promotes cell death in TNBC/BLBC cells in a narrow window of concentrations. There is no previous report on the effect of exogenously administered S1P on TNBC/BLBC. Our previous studies also reported the effect of S1P on the proliferation of MCF7, MDA-MB-361, and MDA-MB 231 cells. These studies showed that S1P induced cell death at higher concentrations[92].

In the proliferation studies (Figure. 4), it was found that treatment of these breast cancer cells with various concentrations of S1P 0.1-20 μM for 72 h inhibited proliferation at high concentrations and showed more proliferative effect at lower concentrations.

Cytotoxicity and proliferation results provide supporting evidence that the effect of S1P is concentration-related[89], as well as the dual action of S1P[76]. These results also suggest that S1P has intracellular and extracellular targets responsible for producing these actions. Different physiological processes are regulated by S1P via its five G-protein coupled receptors[174], and different types of cells have different expression levels of S1PRs[175]. Although the intracellular action of S1P is not yet identified, it seems from the data obtained that intracellular function is independent of the cell surface receptor function. Several reports showed that S1P induced cell survival and proliferation by extracellular mechanisms[176 177]. The result obtained from our proliferation studies are in line with previous research that cell surface S1P receptors promote cell proliferation and survival. However, cytotoxicity studies suggest that external S1P may be transported into the cells. Response at lower concentrations rather than high concentrations can be explained as S1PRs are saturated at higher concentration of S1P and are only activated at nanomolar concentration[178]. The inhibitory effects of S1P are observed at micromolar concentrations, whereas G protein activation is observed at nanomolar concentrations as reported by Davaille[178]. Inhibition of cell migration

by exogenous administration of S1P was reported to be receptor independent and attributed to intracellular mediated mechanism[168].

S1P was found to induce apoptosis in human hepatoma cells (Hep3B) in dose-dependent manner[179]. Another study reported S1P induction of apoptosis in hippocampal neurons[180]. These reports indicate that S1P has anti-proliferative action, yet the exact mechanism is still unknown.

These results may be explained as follows, first depending on the cell type, different cells respond differently via intracellular or receptor-mediated activities. Second, the intracellular action may be affected by different concentrations of S1P, as the exogenously administered S1P has a higher concentration on the outside, whereas the intracellular effects may require less concentration of S1P. Another explanation could be that S1P administered can induce the generation of intracellular S1P, which then can interact with the intracellular target.

In summary, this study shows that S1P has a dual effect on TNBC/BLBC, and the effect is concentration-dependent. Proliferation studies showed that at low concentrations S1P marginally stimulated survival pathway and at higher concentration it exhibited an anti-proliferative effect (Figure 4). Since we have no information about the degradation of exogenously administered S1P and no available information about the expression of S1PRs in these cell lines, and S1PR2 responsible for the anti-proliferative effect S1P[109], I suggest that S1P interact with S1PR2 and producing this effect.

5.2 SPHINGOSINE-1-PHOSPHATE INDUCESES APOPTOSIS IN TRIPLE NEGATIVE/BASAL-LIKE CELL LINES

In this part, I aimed to determine whether the inhibitory effect of S1P is due to apoptosis. The results obtained from this part indicated that S1P significantly reduced cell viability. These results are in line with previous with previous reports [92 128 129 180].

This is the first study to report that S1P induces apoptosis in TBNC/BLBC cells. The exact mechanism is still unknown. However, from previous studies done on different cell lines it was shown that S1P inhibitory action is independent of the S1PR. These results suggest that the proliferation inhibitory action is achieved by intracellular targets. Previous studies pointed that the inhibitory action of S1P is not related to its cell surface receptor by measuring the intracellular concentration of S1P. The intracellular concentration was reported to be higher than in control cells[180]. Shin *et al.* studied the effect of exogenous S1P in melanoma cells. In their experiment, they used Dihydrosphingosine, an S1P analog which activates S1PR, which did not produce the same action of exogenous S1P[176] indicating that the apoptotic effect of S1P is not mediated by cell surface receptors. They reported that the apoptotic effect of exogenous S1P was mediated by caspase-3 activation[128]. Moreover, the apoptotic effect of S1P was not related to metabolic conversion to ceramide as reported by Davaille *et al.*[129]. In all these previous studies, each cell line responded to different S1P concentrations indicating that concentration is cell line specific and S1P concentrations determine its effect.

5.3 SPHINGOSINE-1-PHOSPAHTE AND CHEMOTHERAPY COMBINATION

One of the main objectives of this project is to identify new drug combinations that synergistically reduce the viability of tumor cells. To assess whether the combination of S1P and chemotherapeutic agents doxorubicin and docetaxel potentiate cytotoxic effects in the nine cell lines, cell were treated with either docetaxel, doxorubicin alone or with their combination with S1P for 72 h. Cytotoxicity studies of S1P alone revealed that S1P has a modest effect on cell death. As mentioned above, chemotherapy remains the mainstay of TNBCs due to lack of specific targets. However, the recurrence rate is still high. Neoadjuvant anthracycline, Taxanes are mostly commonly used drugs in the treatment of TNBCs[181]. Doxorubicin is an anthracycline antibiotic that is used for the treatment of different types of tumors. The major mechanism of Doxorubicin action is intercalation into DNA and through the generation of free radicals[182]. Briefly, Doxorubicin enters the nucleus and interact act with the DNA resulting in DNA damage.

Docetaxel belongs to taxanes drug class that acts by stabilizing microtubule assembly[183]. Docetaxel is an anti-microtubules agent that prevents depolymerization of microtubules promoting apoptosis in tumor cells [184]. Docetaxel has several mechanisms of action that is not exclusive to mitosis inhibition. Apoptosis induction by docetaxel is a very complex process involving several transduction pathways.

In the previous experiments, I have identified that S1P causes marked reduction of the viability of apoptosis in TNBC/BLBC cell lines (figure 8). However, the results obtained from combination studies showed no advantage over the chemotherapy alone. A substantial reduction in a number of dead cells was observed in different cell lines. These results raise

the conundrum of how S1P alone caused cell death, whereas in combination with docetaxel or doxorubicin the percentage of cell death in some cell lines was decreased.

The neutralizing responses and reductive responses observed in this combination can attributed to pharmacokinetic and/or pharmacodynamics interactions. Pharmacokinetically, the interaction may be due to decreased permeation and transport of one or both components, increased metabolism and increased the efflux of one or both components of the combination. Tumor microenvironment can influence the action of the chemotherapeutic agent. With the intracellular pH is higher than the extracellular pH, the diffusion of drug into the cell is influenced by the acidic pH of the extracellular environment. Protonation of weak acid drugs such as doxorubicin decreases its uptake[185 186]. The decreased effect of the combination can be due to reduced S1P or the chemotherapeutic agents transport into the cell. Reduction of paracellular permeability due to aggregation, inhibited uptake of both S1P and the drug, or decreased transport of both S1P and its partner drug can be a potential reason for the deacreased effect of the combination. Aggregation reduces the permeability of S1P and or chemotherapy thus results in a reduction of cell death compared with chemotherapy alone.

Competitive inhibition of transporter or target site is also a possible explanation for the observed results. Since the exact mechanism of cellular uptake of docetaxel, doxorubicin and S1P is unknown, a reasonable explanation that pharmacokinetic antagonism at the cellular uptake level may have reduced the uptake of S1P and or the chemotherapeutic agent.

Since the mechanism of S1P uptake into the cell in not well known and the chemotherapeutic drug, doxorubicin and docetaxel uptake into the cell is not fully understood, an assumption is that S1P and chemotherapeutic drug share the same drug transporter uptake mechanism. Both drugs in the combination compete for the same mechanism. Assuming that some of the S1P does not enter the cell when combined with these

drugs, more S1P is available in the extracellular environment and, therefore, producing the opposite action. FTY720, the S1P analog has been shown to inhibit ceramide synthase and S1P lyase upon binding to extracellular receptors[187]. Extrapolating these effect on extracellular S1P, the reduction of the combination can be strongly attributed to these actions.

Reduction in the action of the combination can also be explained by increased metabolism. An assumption that one of the components of the combination increases the metabolism of the other. Supporting this assumption, doxorubicin was also reported to increase the production and secretion of S1P by increasing SphK1 expression [188]. Moreover, SphK1 expression was reported to be upregulated in TNBC[155]. Doxorubicin was also reported to induce expression of SphK1 after 24 treatment in U937 cells [165]. Doxorubicin was also shown to increase S1P secretion through ABCC1 transporter[188]. Nuclear S1P is likely to be involved in cell arrest[189] while extracellular S1P was reported by several studies to enhance cell proliferation[190]. In this regard, rational explanation for the antagonism that the combination prevented the administered S1P from entering the cell, hence, interacting with cell surface receptor and promoting cell proliferation rather than causing apoptosis.

S1P hydroxylase1 (SPP1), an enzyme that terminates the action of S1P [191], modulates intracellular levels of S1P and thus alters the intracellular ceramide levels. Doxorubicin was shown to up-regulate expression of SPP1 in a time-dependent manner[192]. The same study reported that SPP1 downregulation enhanced the apoptotic effect of doxorubicin. Based on these result, I assume that doxorubicin enhanced the expression of SSP1 in these cell lines causing rapid degradation of S1P.

The reduction in number of dead cells in the combination can be due to drug efflux from the cell. Expression of ABCC1 transporter in ER- breast cancer is associated with poor prognosis [193]. Resistance to Doxorubicin treatment involves drug transporters. ABCB1, ATP-

dependent drug pump, acts as drug efflux pump[194]. Other transporters involved in doxorubicin resistance such as ABCC1 (MRP1) ABCC2, ABCC3, ABCG2, and RALBP1[195]. Efflux of doxorubicin from cells has been linked to overexpression of these transporters. S1P is also a substrate for ABCC1, ABCA1, and ABCG2 (also known as breast cancer resistance protein; BCRP)[109]. ABCC1, ABCC11, and ABCG2 were found to be highly expressed in basal-like breast cancer[158] contributing to their aggressive nature and resistant to treatment. Docetaxel efflux is influenced by overexpression of ABCB4 and ABCC1, ABCG2[183].

No information is available yet about the overexpression of the other transporters in the nine cell lines. However, assuming that they are overexpressed in TNBC/BLBC cell lines that demonstrated less response to chemotherapy alone. The overexpression of these transporters may be a strong reason for their responsiveness to treatment. Specific inhibitors of ABC transporters may be used to investigate the effect of these transporters in efflux the combination one of them or both.

The results from combination studies can be attributed to the interference of one of the components of the combination with the target of the partner drug. One example of combination antagonism is that one of the drugs target the related pathway of the other drug in the combination, thus reducing or abolishing the action of the partner drug. Spiegel *et al*. reported that doxorubicin decreased the expression of SphK2[125]. Previous studies have reported that overexpression of SphK2 leads to cell death[122,126]. However, the exact mechanism of this process is still unknown. Assuming that SphK2 is the target of exogenously administered S1P when given in combination with doxorubicin or docetaxel, S1P does not reach its target in sufficient concertation to produce its action. This could be a reason the exogenously administered S1P does not interact with its intracellular target.

S1P may cause alterations of targets involved in apoptosis that are necessary for doxorubicin to produce its effect. Doxorubicin cytotoxicity is achieved by interchelation with DNA and inhibition of Topoisomerase II (TOP2A). Reduced cytotoxicity of doxorubicin in TNBC is strongly associated with TOP2A mutation[196]. Therefore, cell lines that show less response to doxorubicin treatment alone may have TOP2A mutation. Class III beta-tubulin, a protein that make up the microtubules has been associated with increased resistance to Taxanes[68]. MD-MBA-468 was the least responsive to docetaxel treatment alone. No available information about the expression of beta-tubulin in these cell lines. However, overexpression was reported in different tumors [68].

Genetic interaction can influence response to therapy, and hence any disruption in the form of genetic variation (SNPs, Insertion-deletion and copy number variation) will influence how different cells will respond to different treatments. Therefore, examining the changes due to genetic deletion through gene set enrichment analysis provides a rational approach to identify potential differences in response to treatments in different cell lines. Expression2Kinases (X2K) is a free online analysis tool that provides gene set enrichment analysis for any list of genes against known databases of like gene ontology (GO), kinases, transcription factors and pathways(http://www.maayanlab.net/X2K/).

In order to have a better understanding of the disrupted machinery in each cell line, I collected the deleted genes of each of the cell lines I used in my research and previous research done in our lab, then applied gene set enrichment analysis to have a better understanding on what cell functions are mainly influenced in these cell lines.

TNBC/BLBCs have different deleted genes compared to previously used cell lines as shown in Table 3. Listed of deleted genes in each cell line was obtained from cancer cell line encyclopedia (http://www.broadinstitute.org).

Data interpretation of deleted genes for TNBC/BLBC cell lines in comparison to other breast cancer cell lines used in previous research provided minute subtle differences that might correlate to the reasoning behind the lack of response on the cell lines used in this experiment. The output from X2K analysis (Table 4) shows that previous cell lines use (MDA-MB-231, MDA-MB-361, and MCF7), have something in common, which is enrichment of cell migration, cell motility and cell motion seems to be highly associated with the deleted genes list for each. On the other hand, cell lines used in my set of experiment showed a variable gene ontology enrichment that have more kinases and phosphatases activity in common. This suggest that the previous set of experiments might have a better influence on cancer metastasis and the current treatment combinations between S1P and DOX or DOC combination might not be as effective in this case.

Table 3: List of deleted genes in TNBC/BLB, MCF-7, MDA-MB-361, and MDA-MB-231 cell Lines

HCC1599	HCC1143	MDA-MB-468	HCC38	HCC70	HCC1806	HCC1187	DU4475	MCF-7	MDA-MB-231	MDA-MB-361
PDE4DIP	CDK11B	MYST4	CDK11B	PDE4DIP	PDE4DIP	DDR2	CABC1	FMN2	CDK11B	CDK11B
ILK	PDE4DIP	CTBP2	MYO3A	CABC1	DDR2	CABC1	FMN2	CTBP2	TNFRSF1B	ARNT
MAML2	CTBP2	ILK	MYST4	FMN2	FMN2	ILK	FMN2	TCERG1L	MYCL1	ILK
NCAM1	ILK	PIK3C2A	ILK	NLRP3	MYST4	TMEM123	ILK	ILK	PDE4DIP	MAML2
BRCA2	TMEM123	NUMA1	TMEM123	MYST4	NLRP6	NCAM1	MAML2	MAML2	PDE4DIP	TMEM123
STAT5A	TMEM123	TMEM123	NCAM1	PTEN	ILK	FLI1	NCAM1	NCAM1	TPR	NCAM1
ALPK2	NCAM1	NCAM1	SORL1	TNKS2	PIK3C2A	GUCY2C	FLI1	ALPK2	CDC42BPA	FLI1
ADAM17	FLI1	FLI1	FLI1	ILK	TMEM123	TSC2	MAPKBP1	ADAM17	CACNB2	GUCY2C
AAK1	GUCY2C	CSNK1G1	GUCY2C	TMEM123	NCAM1	ACACA	KLK7	AAK1	PCDH15	PIK3C2G
TNRC6B	HSP90B1	ERBB2	PIK3C2G	NCAM1	FLI1	ALPK2	AURKC	NCOA3	ILK	MAF
EP300	MAF	KLK7	MAF	FLI1	PIK3C2G	ADAM17	ADAM17	MYH9	PGR	KLK7
TOP2B	KLK7	ADAM17	BRIP1	GUCY2C	HSP90B1	AAK1	PAX3	MAML3	TMEM123	ADAM17
TEC	AAK1	NCOA3	ALPK2	RB1	MAF	FN1	EP300	VEGFC	MMP1	FN1
MAML3	FN1	TNRC6B	KLK7	BMP4	KLK7	PAX3	MYLK	MAP3K1	NCAM1	PAX3
VEGFC	NCOA3	CNTN6	ADAM17	WDHD1	ADAM17	MYH9	FGFR3	MAP3K4	NCAM1	EP300
MAP3K1	CNTN6	BMP2K	FN1	MLKL	STRADB	MAPKAPK3	MAML3	MET	PCSK7	FGFR3
FLT4	MAPKAPK3	MAML3	PAX3	ALPK2	FN1	FGFR3	VEGFC		SORL1	KDR
MAPK14	MAML3	VEGFC	NCOA3	BAX	PAX3	TEC	CHD1		FLI1	MAML3
ADAM28	VEGFC	MAP3K1	EIF4E	KLK7	CARD10	PDGFRA	CAMK2A		NCAPD3	VEGFC
PREX2	CHD1	CHD1	MAML3	STAT1	TNRC6B	MAML3	ETV1			
RECQL4	EDN1	NOTCH4	VEGFC	FN1	ROBO2	MSH3	CREB3L2		WNK1	MAP3K1
GPR112	BRD2	FANCE	MAP3K1	PAX3	MAML3	EDN1	ADAM28		GUCY2C	CHD1
	TTBK1	GOPC	CREB3L2	REM1	VEGFC	ETV1	RECQL4		PIK3C2G	EDN1
	ETV1	MAP3K4	ADAM28	NCOA3	MAP3K1	PDK4	MLLT3		KRAS	BRD2
	CREB3L2	ETV1	GPR112	CARD10	CHD1	CREB3L2	SMC2		ITPR2	DNAH8
	ADAM28	CREB3L2		MAML3	EDN1	EPHB6	AR		LRRK2	CREB3L2
	RECQL4	EPHB6		MYLK	MAPK14	ADAM28			DYRK2	ADAM28
	GPR112	ADAM28		TEC	ETV1	RECQL4			KSR2	GPR112
		RECQL4		PDGFRA	ADAM28	GPR112			SACS	TRRAP

Table 4: Expression 2 Kinase output for TNBC/BLBC,MCF-7, MDA-MB-361, and MDA-MB-231 cell Lines

MCF-7	**MDA-MB-361**
Cell migration (GO:0016477)	Cell motility (GO:0048870)
Signal transduction (GO:0007165)	Positive regulation of cell migration (GO:0030335)
Cell motility (GO:0048870)	Response to hypoxia (GO:0001666)
Cell communication (GO:0007154)	Regulation of cell size (GO:0008361)
Cytokinesis (GO:0000910)	Positive regulation of cell motion (GO:0051272)
HCC1806	**HCC70**
Regulation of cell size (GO:0008361)	Protein modification process (GO:0006464)
Positive regulation of kinase activity (GO:0033674)	Positive regulation of caspase activity (GO:0043280)
Positive regulation of protein kinase activity (GO:0045860)	Phosphate metabolic process (GO:0006796)
Positive regulation of transferase activity (GO:0051347)	Protein amino acid phosphorylation (GO:0006468)
Cell migration (GO:0016477)	Peptidyl-tyrosine phosphorylation (GO:0018108)
DU4475	**HCC1887**
Cytokinesis (GO:0000910)	Positive regulation of cell migration (GO:0030335)
Cell division (GO:0051301)	Positive regulation of cell motion (GO:0051272)
Positive regulation of transcription factor activity (GO:0051091)	Regulation of cell size (GO:0008361)
Positive regulation of DNA binding (GO:0043388)	Cell migration (GO:0016477)
Positive regulation of binding (GO:0051099)	Regulation of cell migration (GO:0030334)
MDA-MB-468	**MDA-MB-231**
Positive regulation of kinase activity (GO:0033674)	Positive regulation of cell migration (GO:0030335)
Positive regulation of protein kinase activity (GO:0045860)	Positive regulation of cell motion (GO:0051272)
Positive regulation of transcription (GO:0045941)	Cell migration (GO:0016477)
Positive regulation of transferase activity (GO:0051347)	Positive regulation of metabolic process (GO:0009893)
Positive regulation of gene expression (GO:0010628)	Regulation of cell migration (GO:0030334)
HCC143	**HCC38**
Regulation of cell size (GO:0008361)	Sensory perception of sound (GO:0007605)
Cell migration (GO:0016477)	Cell migration (GO:0016477)
Spermatogenesis (GO:0007283)	Positive regulation of metabolic process (GO:0009893)
Eicosanoid biosynthetic process (GO:0046456)	Positive regulation of transcription (GO:0045941)
Regulation of blood vessel size (GO:0050880)	Positive regulation of gene expression (GO:0010628)
HCC1599	
Signal transduction (GO:0007165)	
Cell communication (GO:0007154)	
Protein kinase cascade (GO:0007243)	
Response to hypoxia (GO:0001666)	
Response to organic substance (GO:0010033)	

6. CONCLUSION AND FUTURE DIRECTIONS

The major objective of this project was to investigate whether S1P can induce cytotoxicity in TNBC/BLBC cell lines. Our data demonstrated that S1P could induce cell death after prolonged exposure in some of the cell lines. Cytotoxicity occurred at a narrow range of S1P concentration.

In proliferation studies, it was found that prolong exposure of S1P can have a marginal proliferative effect. The reason for these contradictory results is not clear, however, this is an indication that S1P has intracellular and extracellular targets.

The mechanisms by which S1P induces cell death was observed to be due to apoptosis at tested concentrations. The images showed that S1P induced morphological changes, shrinkage, and detachment of cells after 72 hr of treatment.

The study also aimed to examine the co-administration of S1P with currently used chemotherapeutic agents. In this part, results obtained showed a different response than the previous studies. The combination showed no significant advantage over the chemotherapeutic agents alone and in some cell lines, it attenuated the effect of the drug itself.

We need further investigation about the downstream pathways that triggered an apoptotic effect in TNBC/BLBC. An evaluation of the expression of cell surface S1PRs and the S1P metabolizing enzyme is eagerly needed to understand better the morphological changes after the treatment. This can be done by extracting mRNAs for the S1PRs from TNBC/BLBC cell lines and performing quantitative RT-PCR. Assessing the expression of S1PR can give more explanation of results obtained from this study.

Pharmacological studies using S1PR antagonist maybe used for further understanding of S1P function. Several S1PR receptors antagonists are currently available such as VPC23019, which is an S1PR1 antagonist that can be used to block the function of S1PR1, and therefore we can test the effect of exogenous S1P on these cell lines. Receptor down regulation is another approach for understanding the function. Gene deletion for specific S1PR can exclude that the receptor product of the deleted gene mediates an effect. Molecular approaches to use microRNA or siRNA to regulate gene expression for S1PR or enzyme involved in S1P pathway can also be used to understand the different response of TNBC/BLBC to exogenous S1P. Labeled S1P can be used to clarify whether the exogenously administered S1P enters the cell and S1P trafficking.

Further investigation is needed to understand the lack of response in the chemotherapy combination studies. Interaction of S1P with chemotherapeutic agents or expression level of efflux transporter, P-gp, could explain the results obtained.

Based on X2K result, I would suggest that combination with tyrosine kinase inhibitors might potentially show a more significant synergistic effect when combined to S1P in TNBC/BLBC cell lines.

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